DEOXYVARIOLINS AND POLYMER THERAPEUTICS

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"Knowledge is proud that he has learned so much;
Wisdom is humble that he knows no more."

- William Cowper

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CONTENTS

ABSTRACT

Polymeric carrier molecules have been shown to improve the pharmacokinetics and pharmacological profile of small-molecule anticancer drugs. The variolins are a group of marine natural product-derived cytotoxins whose clinical efficacy may be improved through conjugation with a polymer backbone.

This thesis first describes the optimisation of a synthesis of the non-natural analogue deoxyvariolin B, a synthesis that was devised by Anderson and Morris immediately prior to the commencement of this project. The synthesis, comprised of six linear steps, was refined to give an overall yield of 25%.

As part of a much larger structure-activity relationship investigation being carried out by Pharma Mar, SA (a Spanish pharmaceutical company specialising in marine natural products), a small library of deoxyvariolin analogues, numbering approximately fifteen, was also synthesised and tested *in vitro* against the P388 murine leukaemia cell line. All analogues synthesised showed an appreciable loss of bioactivity (typically around an order of magnitutude) compared to that of deoxyvariolin B.

Attempts at acylating deoxyvariolin B with the tetrapeptide biolinker were hampered by DVB's awkward reactivity and the low stability of some of the products. A more suitable deoxyvariolin analogue, in terms of its bioactivity as well as chemical reactivity, was therefore chosen for development into a polymer therapeutic. This analogue was successfully conjugated with a tetrapeptide biolinker to give a compararably bioactive conjugate that was to be reacted with an activated polymer backbone.

The final part of this thesis, however, describes work that was carried out with the polymeric starting material, in which it was revealed that two unforeseen side reactions were taking place. These side reactions ultimately precluded the synthesis of the target constructs.

ABBREVIATIONS, ACRONYMS AND UNITS

Å	angstrom(s)
β-	*beta-*
δ	chemical shift in parts per million
υ_{max}	absorbance maxima in cm^{-1}
ε	extinction coefficient
°C	degrees celcius
μ	micro
1A2P	1-amino-2-propanol
1D	one-dimenional
2D	two-dimensional
AcCl	acetyl chloride
AMP	4-aminomethyl piperidine
Ar	aryl
ATP	adenosine triphosphate
ATRP	atom-transfer radical polymerisation
AU	absorbance units
aq	aqueous
br	broad (in NMR)
Bu	butyl
c	centi
calcd	calculated
CDK	cyclin-dependent kinase
CIGAR	constant time inverse-detection gradient accordion rescaled long-range heteronuclear multiple bond correlation spectroscopy
conc	concentrated
COSY	correlation spectroscopy
CPDB	2-(2-cyanopropyl)dithiobenzoate
d	doublet (in NMR)

Da	daltons
DCC	dicyclohexylcarbodiimide
DCU	dicyclohexyl urea
dec.	decomposes
DEPT	distortionless enhancement by polarization transfer
dil	dilute
DIOL	dialcohol phase (in chromatography)
DMAP	4-(dimethylamino)pyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DVB	deoxyvariolin B
EDCI	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EPR	enhanced permeability and retention
ESI	electrospray ionisation
EtOAc	ethyl acetate
EtOH	ethanol
eq	equivalents
F	L-phenylalanine
Fmoc	9-fluorenylmethoxycarbonyl
FTIR	fourier transform infra-red
g	gram(s)
G	glycine
GFLG	glycyl-L-phenylalanyl-L-leucyl-glycine
GPC	gel-permeation chromatography
h	hour(s)
HBTU	*O*-benzotriazole-1-yl-tetramethyluronium hexafluorophosphate
HOBT	1-hydroxybenzotriazole
HPLC	high-pressure liquid chromatography
HPMA	*N*-(2-hydroxypropyl) methacrylamide
HRMS	high-resolution mass spectrometry
HSQC	heteronuclear single quantum coherence
Hz	hertz

i-	*iso-*
IC_{50}	inhibitory concentration (50%)
IPA	*iso*-propyl alcohol
J	coupling constant
k	kilo
L	L-leucine or litre(s)
LCMS	liquid chromatography with mass spectrometric detection
lit	literature
LRMS	low-resolution mass spectrometry
m	multiplet (in NMR) or milli (as a unit prefix) or metre(s)
M	molar
m-CPBA	*meta*-chloroperbenzoic acid
MeOH	methanol
min	minute(s)
mol	mole(s)
mp	melting point
MS	mass spectrometry
m/z	molar mass to charge ratio
n	nano
n-	*normal-*
NHS	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance
OAc	acetate
OBn	*O*-benzyl
p-	*para-*
PD	polydispersity
PEG	poly(ethylene glycol)
pHPMA	poly[*N*-(2-hydroxypropyl) methacrylamide]
pMAC	poly(methacryloyl chloride)
pMAOS	poly(methacryloxysuccinimide
PMB	*para*-methoxybenzyl
pMMA	poly(methyl methacrylate)
pNPMA	poly(*para*-nitrophenyl methacrylate)

ppm	parts per million
Pr	propyl
q	quartet (in NMR)
RAFT	reverse addition fragmentation
rt	room temperature
SAR	structure-activity relationship
s	singlet (in NMR)
sat	saturated
SEC	size-exclusion chromatography
SMe	thiomethyl
t	triplet (in NMR)
t-	*tertiary*
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
tlc	thin-layer chromatography
UoC	University of Canterbury
UV	ultraviolet (*adj.*) or ultraviolet light (*n.*)

CHAPTER 1

INTRODUCTION

1.1 Cancer

A report published in 2003 by the World Health Organisation[1] stated that over 10 million people are diagnosed with cancer and over 6 million die from the disease annually (at the time of writing, the web pages of Cancer Research UK[2] report the figures as 10.9 million and 6.7 million, respectively). Globally, there are marked regional differences in the occurrence and type of cancers. Western societies bear the greatest cancer burden, with tumours associated with smoking and Western lifestyles (specifically, cancers of the lung, colorectum, breast and prostate) being the most common. In Western Europe and the US, cancer is the leading disease cause of death in women between the age of 40 and 60 and is second only to heart attack as a cause of death in males over 30.[3] In developing countries, up to 25% of tumours are associated with chronic infections, such as hepatitis B virus (liver cancer), human papillomaviruses (cervical cancer) and *Helicobacter pylori* (stomach cancer).

The global incidence of and mortality from cancer increased by just under 20% in the decade from 1990 to 2000.[1] Furthermore, cancer is closely related to the ageing process, and so is set to become more common worldwide as more people live to an older age.[3]

1.1.1 Cancer biology

The term *cancer* represents a group of diseases, all characterised by uncontrolled cell growth. The key cellular events in the progress of cancer include the loss of proliferative control, the failure to undergo apoptosis (programmed cell death), neoangiogenisis (the growth of new blood vessels), tissue remodelling, invasion of tumour cells into surrounding tissue and, finally, metastatic dissemination of tumour cells into distant organs.[4] The process of malignant transformation is a multi-step one, requiring the sequential acquisition of genetic alterations. The transition from normal to malignant growth can sometimes take over 20 years.[1]

In a typical tumour, the cancer cells often occupy less than half of the tumour volume, blood vessels 1-10%, and the remaining volume – termed the *interstitium* – being comprised of a collagen-rich matrix bathed in interstitial fluid. It is through this fluid that oxygen, metabolites and bioactive compounds must travel in order to reach and be absorbed by cancer cells.[5]

1.1.2 Cancer treatment

Surgical removal or irradiation of tumours remain the first choices of treatment, with chemotherapy being relied upon if a tumour has metastasized, or if parts of the primary tumour cannot be removed.[5] Non-selective classical anticancer drugs usually display a broad toxicity towards all dividing cells, with cancer cells generally dividing more rapidly than normal, healthy cells. This non-specific mechanism of toxicity therefore causes the death of healthy, as well as cancerous, tissue, leading to side effects which typically include mucositis (inflammation of a mucous membrane), hair loss, myelosuppression (suppression of the production of blood cells and platelets by the bone marrow) and various types of organ damage.[6]

Small cytostatic drugs will distribute readily throughout most body tissues, with a rapid diffusion across plasma membranes. Better specificity, achieved through the use of drug delivery systems, may therefore help to circumvent the indiscriminate toxicity of existing drugs, and would represent a significant improvement on existing treatment regimes.

1.1.2.1 Barriers to drug delivery

Chemotherapeutic agents are usually administered intravenously. However, the abnormal physiological architecture and properties of tumours lead to some serious limitations regarding the delivery of drugs via the blood supply.[5] Some of these limitations are particularly relevant for the type of construct that is the target of the work described in this thesis (see Section 1.2.2.6), and so will be briefly discussed here in more detail.

There are several important limiting factors in the diffusion of drugs to tumours, first and foremost of which is the heterogeneous, slower and intermittent blood flow that is typically observed within tumour tissue.[7,8] This, of course, leads to non-homogenous distribution within the tumour of intravenously-administered drugs.

A second barrier to drug delivery is the higher hydrostatic pressures present within tumours.[9,10] In normal, healthy tissue the hydrostatic pressure within the interstitium is significantly less than that within the vasculature. This pressure gradient ensures a flux of fluid (carrying dissolved solutes) out of the blood and through the interstitium, with the lymphatic drainage maintaining the pressure gradient. In tumour tissue, however, where numerous vascular and lymphatic anomalies occur,[9,11-13] the interstitial pressure is elevated, uniform throughout the tumour (falling away sharply at the tumour periphery), and roughly equal to the microvascular pressure.[5,10] This lack of a pressure gradient results in greatly reduced fluid flow,* which in turn all but eliminates drug transport across capillary walls by the convective mechanism. Drugs must therefore be transported into the interstitium by simple diffusion, which is a much slower process than convection-based transport, particularly with respect to larger molecules.[5,9]

Poor lymphatic drainage within tumours, leading to accumulation of molecules in the interstitium, also reduces the osmotic gradient that is present in healthy tissues.[9] This will further impede extravasation of macromolecules by diffusion in tumours. However, Jain notes that, despite these limitations, the "large microvascular permeability and interstitial diffusion coefficients of macromolecules in tumours compared to those in normal tissues provide a powerful rationale for the use of large molecular weight agents instead of conventional small molecular weight agents in cancer detection and treatment".[9]

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* There is still a small fluid flux through the tumour, as is evidenced by the observation that ~10% of the blood fluid leaving a solid tumour 'seeps' out from its periphery rather than draining via a vein.[5]

1.2 Polymer Therapeutics

1.2.1 Brief overview of field

The term *polymer therapeutics* is an umbrella term referring to several classes of compounds, including rationally-designed macromolecular drugs and prodrugs, polyplexes for DNA delivery, polymer-drug conjugates, polymer-protein conjugates and polymeric micelles containing covalently bound drug.[14] Polymer therapeutics will alter the biodistribution and/or pharmacokinetics of a drug, and so can be used to target a range of diseases. This potential to alter the pharmacological profile of a drug is particularly important in the treatment of solid tumours, reflected in the fact that 20 out of 32 drug delivery systems currently in use or in clinical trials target cancer.[15]

Though contemporary polymer synthetic chemistry is yielding more structurally complex polymers that hold promise as carrier molecules, all polymer therapeutics that have been transferred to the clinic to date have been based around linear, random coil polymer structures. These polymers include synthetic polymers [poly(ethylene glycol) (PEG), *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers, poly(vinylpyrrolidone), poly(ethyleneimine), linear polyamidoamines and pyran copolymers], natural polymers (dextran, dextrin, hyaluronic acid and chitosans) and pseudosynthetic polymers [man-made poly(amino acids)].[14] Figure 1.1 gives the structures of some representative examples of these polymers.

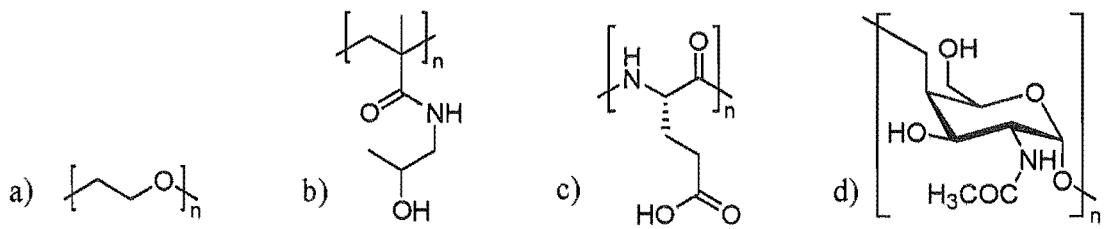


Figure 1.1: Examples of polymers used as carrier molecules for drugs; a) PEG, b) pHPMA, c) poly(glutamic acid), and d) *N*-acetyl-α-1,4-polygalactosamine.

This thesis describes work towards the synthesis of linear polymeric constructs based around pHPMA and carrying anticancer cytotoxins.

1.2.2 Polymer-drug conjugates

In the 1970s De Duve recognised the potential for lysosomotropic drug delivery by macromolecules and site-specific intralysosomal drug liberation.[16] Ringsdorf extended this idea in the same decade to propose an idealised structure for polymer-drug conjugates.[17] A viable mechanism to allow tumour-targeting of these proposed macromolecular prodrugs was not known at the time, other than the observed increased rates of endocytosis of cancer cells compared with normal cells.[18] It would not be till several years later, with the important discovery of the *enhanced permeability and retention effect* (EPR effect, see below), that a promising mechanism of drug targeting would be revealed.

1.2.2.1 The EPR effect

The EPR effect (Figure 1.2) leads to the accumulation of macromolecules in tumour tissue.[19] The phenomenon was first described by Matsumura and Maeda,[20] following investigations involving SMANCS (a polymer-protein conjugate incorporating the antitumour protein neocarzinostatin) carried out by Maeda and colleagues,[21] and is thought to be due to two synergistic features of tumour tissue. The first is the hyperpermeability of tumour blood vessels which, unlike healthy vasculature, leads to extravasation of macromolecules into the tumour interstitium.[9] The second is the very poorly developed lymphatic system typically seen within tumours, which leads to accumulation of the macromolecules within the tumour.[12,13,21]

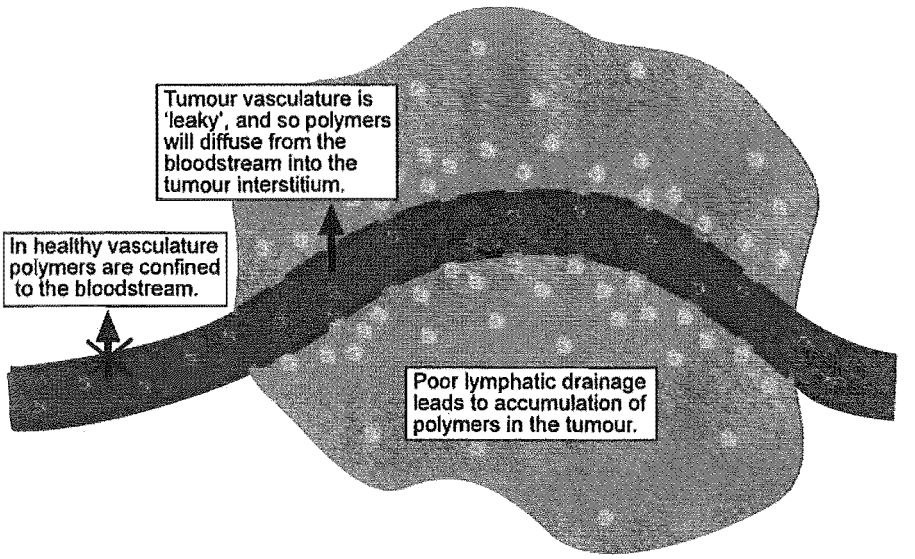


Figure 1.2: The EPR effect.

1.2.2.2 Site-specific drug liberation

As was highlighted by de Duve and Ringsdorf 30 years ago,[16,17] a crucially important feature of a polymer-drug construct is the moiety used to conjugate the drug onto the polymer backbone. This group needs to be stable in the blood plasma (and urine) yet cleaved at the site of action. It is important to optimise this reactivity, as a linker that is not cleaved at the site of action will permanently mask the therapeutic cytotoxicity of the bound drug, while one that allows premature drug liberation will lead to indiscriminate toxicity.[22]

Following uptake by cancer cells through pinocytosis, polymer-drug constructs will be enclosed within *lysosomes* - intracellular vesicles possessing an acidic pH of 4-5 (typically around 4.8, though values as low as 4.0 have been measured).[23,24] Also present in lysosomes are a group of cysteine protease enzymes named the *cathepsins*. Either the drop in pH (from a blood plasma value of ~7.4) or the presence of this group of proteases can facilitate liberation of the active drug from the polymer backbone.

The first of these mechanisms - acid-catalysed drug liberation - can be utilised through the use of acid-sensitive linker groups to conjugate the drug onto the polymer. Examples include *cis*-aconityl, acetal and hydrazone linkages (Figure 1.3).[14] The latter of these has been used in the construction of a pHPMA-doxorubicin conjugate, which shows superior activity over PK1 (see Section 1.2.2.6 for a description of this prototypical polymer-drug conjugate) against lymphoma *in vivo*.[25]

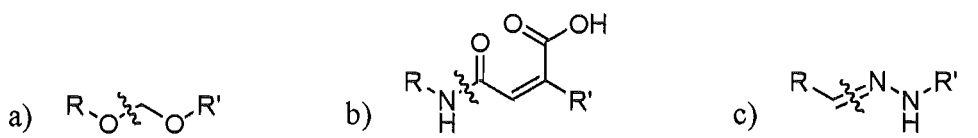


Figure 1.3: Examples of acid-sensitive linker groups, with the site of cleavage shown; a) the acetal, b) the *cis*-aconityl, and c) the hydrazone groups (the imine bond in the latter is replaced by an aldehyde group in the cleavage product).

A variation on this theme employs a polyacetal backbone, so that acid-catalysed acetal hydrolysis gives an acylated bioactive analogue of the drug (carrying the non-biodegradable linker).[26]

As mentioned above, an alternative to the acid-catalysed route to drug liberation is the cathepsin-mediated cleavage of peptide bonds. By analogy with the polyacetal backbone example, this can be capitalised on by using biodegradable cathepsin-cleavable polymers. An example of one such construct is the prodrug CT-2103,[27] a promising paclitaxel-carrying polymer-drug conjugate currently in development that is based around poly(glutamic acid) – a polymer that is cleaved by cathepsin B to liberate a drug-glutamic acid conjugate from which the free drug is then liberated by non-enzymatic ester hydrolysis.[28]

Another well-established technology employs a non-biodegradable polymer carrying the tetrapeptide biolinker glycyl-L-phenylalanyl-L-leucyl-glycine (GFLG, Figure 1.4), a motif that is stable in circulation[29] but cleaved intralysosomally by cathepsins B, H and L.[30]

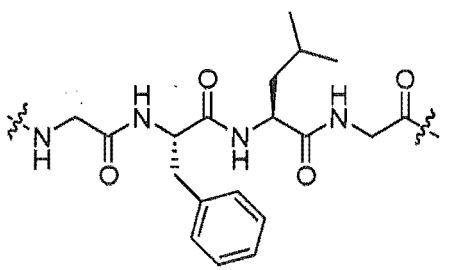


Figure 1.4: The biodegradable tetrapeptide motif, GFLG.

Antitumour drugs bound to a polymeric carrier via this biolinker will therefore be liberated intracellularly at their site of action, given that the EPR effect ensures accumulation of such polymeric prodrugs in the tumour interstitium and that macromolecules are inherently lysosomotropic.[16] It is this mechanism of drug targeting and site-specific liberation that was chosen in the work described in this thesis.

Finally, a related strategy in the early stages of development, termed polymer-directed enzyme prodrug therapy (PDEPT), involves the administration of two separate polymeric constructs– the first one bearing drug bound via a biodegradable linker, and the second, administered a short time later, carrying an enzyme which will cleave the biodegradable linker. Proof of concept was achieved *in vivo*, firstly with an HPMA-GFLG-doxorubicin copolymer combined with an HPMA-cathepsin B copolymer,[31] and secondly with an HPMA copolymer bearing doxorubicin and cephalosporin, both bound via a glycylglycine dipeptide linker, combined with an HPMA copolymer-β-lactamase conjugate.[32] With the drug being liberated in the tumour interstitium,

diffusion rates will be higher[5] and some types of drug resistance of tumour cells (specifically, that based on reduced rates of pinocytosis) may be overcome.

1.2.2.3 Further discussion on pharmacokinetics and pharmacology

The most obvious additional advantage of conjugating drugs onto polymers is that the toxicity of the drug is masked while in conjugate form. Assuming, then, that the biolinker is stable in both blood plasma and urine, this will lead to much lower levels of haemolysis and bladder toxicity, respectively. And to reiterate what has already been mentioned (1.2.2.1), indiscriminate toxicity towards other tissues and organs is avoided due to the vascular-localisation afforded by the size of the polymer constructs.

Further advantages include the potential to increase the water solubility of drugs. Taken together with the above advantages, this will give superior pharmacokinetics of polymer-drug constructs over the free drugs. Specifically, higher plasma concentrations of a drug can be achieved (without the associated toxicity that would otherwise be observed with the free drug), with longer plasma residence times due to slower renal excretion.[33] If effective targeting through the EPR effect can be achieved, the low toxicity of a polymeric prodrug will give a superior pharmacological profile compared with free drug.

1.2.2.4 Targeting ligands

In addition to the passive targeting afforded by the EPR effect, the potential also exists for receptor-mediated targeting through the attachment of tumour-targeting ligands on the polymer backbone.[17] However, due to the biochemical similarity between malignant and healthy cells, few ligand/receptor combinations have been identified. A further complication is the genetic instability seen in rapidly growing tumours, commonly leading to the appearance of 'antigen loss variants'.[1] These resistant variants will be selected for through the use of chemotherapeutic strategies employing active targeting mechanisms. It is for these reasons that no polymer therapeutics bearing tumour-targeting ligands have thus far progressed into clinical development.[14]

The potential does exist, however, for more general active targeting mechanisms to be employed. One such example is PK2, the first polymer-drug conjugate bearing a targeting ligand to be clinically tested.[34] It is a galactosamine-bearing analogue of PK1 (see Section 1.2.2.6), designed

to target the asialoglycoprotein (ASGP) receptors of hepatocellular carcinomas (though healthy ASGP receptor-bearing hepatocytes are also targeted).[35] Clinical trials involving human patients and gamma camera imaging confirmed 15-20% dose targeting of PK2 to the liver after 24 h.[36,37]

1.2.2.5 pHPMA

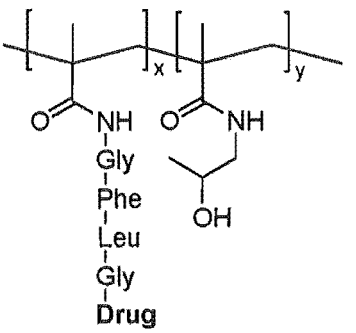
Most anticancer polymer-drug constructs tested clinically have been based around pHPMA (see Figure 1.1b),[14] a polymer originally developed by Kopecek and colleagues as a plasma expander.[38,39] This is a highly water soluble and biocompatible polymer possessing very low, non-clinically relevant immunogenicity.[40]

A 2003 review points out that most of the pHPMA copolymer constructs reported thus far have incorporated traditional small-molecule anticancer drugs.[6] It goes on to summarise that experimental studies on pHPMA constructs carrying the anticancer drugs daunomycin, doxorubicin, farmorubicin, methotrexate and 5-fluorouracil showed a combination of markedly improved antitumour activity, increased maximum tolerated dose (relative to free drug), activity towards multidrug resistant cells, reduced myelotoxicity, hepatotoxicity, cardiotoxicity, nephrotoxicity and toxicity to thymus, prolonged circulation in the bloodstream and enhanced accumulation in solid tumours.

1.2.2.6 The target construct

This thesis describes work towards the synthesis of polymer therapeutics of type **1.1**, based around pHPMA and carrying anticancer drugs bound via the tetrapeptide motif GFLG (Figure 1.5). No attempts were made to include targeting moieties.

There are many published accounts of the synthesis and biological activity of constructs of type **1.1**. The most well-documented is the prototypical PK1, a doxorubicin-carrying polymer-drug conjugate of ~28 kDa.[41] Doxorubicin is an anticancer drug widely used in the treatment of both solid tumours and leukaemia, though the clinical use of this chemotherapeutic is limited by its indiscriminate toxicity.[42] PK1 displays a more favourable pharmacological profile compared with that of free doxorubicin, and is currently in phase II clinical trials.[43]



1.1

Figure 1.5: A generalised polymer-drug conjugate of type **1.1**.

PK1 has been extensively studied, with a large amount of published data regarding its *in vivo* biological properties in both mouse models and human patients. It was the first polymer-drug conjugate to enter phase I clinical trials as an anticancer agent, during which time it was found that it gave a four- to five-fold increase in the maximum tolerated dose of bound doxorubicin, a prolonged plasma half-life, an absence of liver accumulation and effective renal elimination. As part of the same study radionuclide imaging results were able to confirm uptake at known tumour sites by 6 out of 21 patients, with two 'partial responses' and two 'minor responses' being observed. Dose-limiting toxicities were typical of anthracyclines and no cardiotoxicity (a well documented side effect of doxorubicin chemotherapy) was observed.[44] Results from a phase II clinical trial involving 63 patients showed that side effects were manageable and the treatment was 'tolerable', though only 6 of the patients showed a positive response.[43] No polymer-related toxicity was detected.

Examples of other conjugates of type **1.1** include the doxorubicin-carrying conjugate PK2 (see Section 1.2.2.4); a construct incorporating cisplatin, which when tested *in vivo* in mouse models showed a more favourable pharmacological profile than the free drug alone;[45] a conjugate carrying the ellipticine derivative 6-(3-aminopropyl)-ellipticine (APE), which when tested in mouse models gave comparable results to free APE;[46] a paclitaxel-bearing conjugate which showed low toxicity in humans (however, the clinical trial was halted due to the severe neurotoxicity observed in parallel studies involving mouse models, possibly indicating premature drug liberation);[47] a construct incorporating the anticancer fungal metabolite wortmannin, which has demonstrable activity *in vitro* and *in vivo* and is in the early stages of development;[48] a construct carrying the antiangiogenesis fumigillol TNP-470, which gave an enhanced and prolonged effect of this broad-spectrum anticancer agent whose use is normally limited by

neurotoxicity at higher doses (the construct did not cross the blood-brain barrier);[49] and finally, a polymer-bound cathepsin K inhibitor, which is in the early stages of development and is to be used in the treatment of osteoporosis and rheumatoid arthritis.[50]

1.2.2.7 Polymer size and pharmacokinetics

Polymer size is crucially important in determining the pharmacokinetics of macromolecular prodrugs. The polymers must be large enough to ensure vascular localisation in healthy vasculature and to prevent rapid renal excretion, but must also be small enough to allow slow renal excretion over a few days.

It is possible to circumvent the upper limit imposed by the renal threshold for glomerular filtration through the use of biodegradable polymers, for example polyacetals and poly(amino acids) (see Section 1.2.2.2 for the mechanisms of biodegradation).[26,28] Upper size limits may still exist, however, as is highlighted by the observation that pHPMA molecules larger than 80 kDa accumulate in the skin,[51] which is clearly an undesirable property for a cytotoxic prodrug.

A further pharmacokinetic consideration when assessing optimal polymer size, and one that receives little attention in the existing polymer therapeutic literature, relates to the rates of prodrug transport within the tumour interstitium. Specifically, larger molecules will take much longer, once they have reached the tumour, to diffuse through the interstitium (which is significantly more extensive in tumour tissue than in healthy tissue) to the target cancer cells (see Section 1.1.2.1 for a more detailed discussion of this phenomenon). Calculations (based on experimental observations) made by Jain provide an illustrative example. He calculated that a 150 kDa monoclonal antibody (corresponding in size to pHPMA sample of approximately 40 kDa) supplied constantly to a tumour one centimetre in diameter with no blood supply to its core would take several months to reach a uniform concentration within the interstitial fluid.[5] This highlights the significance of the distinction between tumour localisation (which is what is generally measured)[51] and cellular uptake of macromolecules.

Thus, two opposing effects will dictate the optimum size for polymeric prodrugs. Namely, accumulation of macromolecules in tumour tissue will improve with increasing prodrug size, while rates of diffusion through the tumour interstitium to the target cells will be decreased. It is therefore necessary to carefully tailor the size of non-biodegradable polymeric carrier molecules to optimise their pharmacokinetic properties.

It should be noted here that hydrodynamic radii, and therefore pharmacokinetic properties, may vary considerably between different molecules of the same size. The random-coil linear polymer PEG, for example, has a hydrodynamic radius some 5-10 times larger than that of a globular protein of equivalent molecular weight.[14] Similarly, pHPMA, also a flexible linear polymer, behaves on GPC as proteins sized 3-6 times as large.[52] Rates of renal excretion may also vary between macromolecules of the same size, depending on the degree of flexibility of the polymer.[52]

As pHPMA is the polymeric carrier molecule that was employed in the work described in this thesis (see Section 1.2.2.4), literature regarding the pharmacokinetics of this polymer is the most relevant here. Looking specifically at pHPMA, then, lower and upper limits for molecular weight have been determined by *in vivo* experiments.[51,52] Polymers less than ~20 kDa are excreted too rapidly, while those above ~70 kDa are excreted too slowly.[52] Tumour accumulation is seen to increase linearly with size up to about 75 kDa.[51] Based on these results,* and considering that polymer samples will always possess some degree of *polydispersity* (defined in Section 4.1.1), it can be concluded that pHPMA sizes of around 60 kDa would be optimal (although one experiment conducted *in vivo* with mouse models even showed a pHPMA-based polymer conjugate of 115 kDa as having a superior pharmacological profile over similar constructs of around 30 kDa).[25] It is therefore unclear as to why 30 kDa is generally quoted as being the optimal size for pHPMA-based anticancer polymer-drug conjugates,[14] nor why this molecular weight was chosen for the numerous clinical trials involving pHPMA-based polymer-drug conjugates carried out to date.[34,43,49] As would be expected for a prodrug of this size, within 60-90 minutes of intravenous administration of PK2 ($\bar{M}_n$ = 27 kDa, PD = 1.4; see Section 1.2.2.4), half of it had been cleared from the blood plasma as the intact polymer-drug conjugate through renal excretion.[37]

Of course, human physiology is complicated and, in the case of tumours, often varied, and so the idea that a single molecular weight for a polymeric delivery molecule will be ideal for all treatments is an oversimplification. Results obtained from investigations carried out *in vivo*, especially those involving human patients, are ultimately the most informative on the efficacy of

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* It should be noted that the results obtained for the size-dependence of tumour accumulation of pHPMA differ considerably from those obtained with other macromolecules.[19,53]

therapeutics. In the development of new therapeutics, established parameters and methodologies should be employed wherever possible, to allow a more accurate comparison with existing technologies. For this reason, a pHPMA molecular size of 30 kDa, which, as has already been mentioned, is approximately that of PK1 and PK2 (see Sections 1.2.2.4 and 1.2.2.6), was considered desirable for the polymeric constructs that were the target of the work being described in this thesis.

1.2.3 Synthetic protocols

Polymer synthesis presents specific challenges for pharmaceutical development. While a synthetic drug or prodrug should be homogenous and composed of a single, defined species, polymers are usually heterogeneous and can be difficult to characterise. Specifically, a polymer sample will contain individual molecules of different molecular weight. The distribution of this molar mass, referred to as the polydispersity (PD, defined in Section 4.1.1), will affect the biodistribution, and therefore the biological activity, of the pharmaceutical agent.

Polymer samples of low PD can be obtained either by size exclusion chromatography (SEC) on polymers synthesised by conventional free radical protocols, or through the use of controlled polymerization protocols. The former is the conventional (though seldom used) method of size control in polymer therapeutic research, while the second is relatively new methodology that was explored in the work described in this thesis (see chapter 4). Both are discussed in more detail below.

1.2.3.1 Conventional synthetic protocols

The synthetic route to polymer-drug conjugates that was developed by Kopecek in 1977[53] dominates all published synthetic accounts to date.[25,37,40,43,46,49,54-57] In these protocols, free radical polymerisation gives an HPMA-based copolymer precursor of relatively high PD, **1.2** (Figure 1.6), or an equivalent precursor, which can then be reacted with a nucleophilic drug to give a construct of type **1.1**. Laborious size fractionation using SEC can be used to give a polymer of PD ~1.2,[52] though this has not been employed in any of the reported polymer-drug conjugate syntheses to date. PDs of the synthesised polymers are often not given,[22,25,40,54,58] but

those that are range from 1.4[46,59] to 1.7.[49,55] Polymer sizes range from 19 kDa to 31 kDa, with the only notable exception being a construct of 115 kDa.[25]

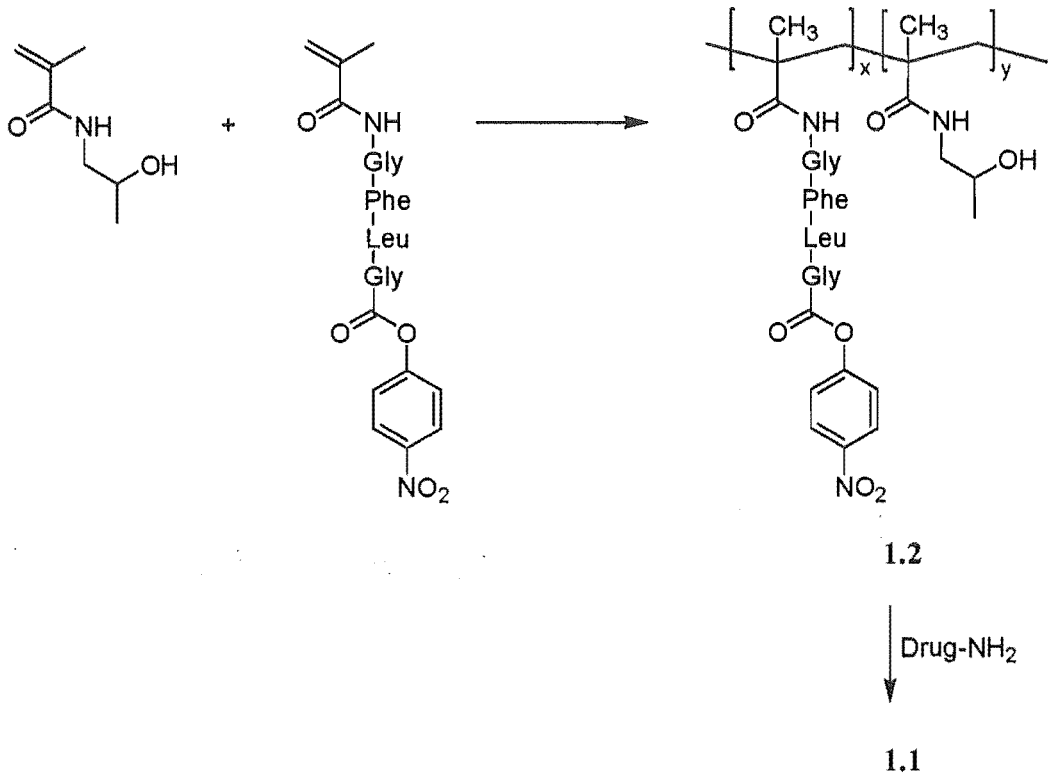


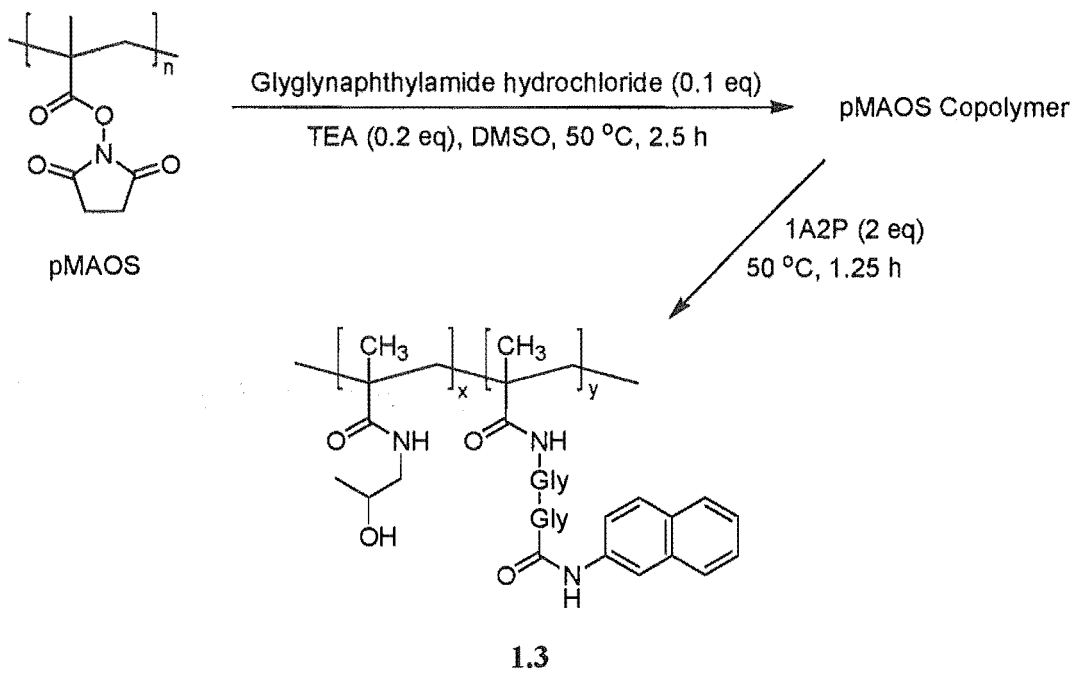
Figure 1.6: Conventional synthetic route to polymer-drug conjugates of type 1.1.

A related mechanism involves the copolymerisation of HPMA with the methacryloyl-tetrapeptide-drug unit to give the polymer-drug conjugate in one step.[48] Of course, the drug must be stable under the polymerisation reaction conditions.

1.2.3.2 pMAOS as a precursor

An alternative synthetic route to pHPMA-based polymer-drug conjugates involves the use of an 'activated' homopolymer backbone, which is functionalised using appropriate nucleophiles. The polymeric activated ester poly(methacryloxysuccinimide) (pMAOS, Figure 1.7) is a relatively hydrolytically stable polymer that was first considered as a possible precursor in the synthesis of polymer therapeutics by Ringsdorf in his seminal 1975 paper.[17] The interest in this polymer as a precursor in the synthesis of polymer-drug conjugates was greatly increased with a 2001 publication by Godwin *et al.*,[60] which reported on the synthesis of pMAOS samples of PD <1.2 using controlled radical polymerisation protocols. Furthermore, the pMAOS was then reacted

with glycyl-glycyl-β-naphthylamide, followed by 1-amino-2-propanol (1A2P) to reportedly give the naphthylamide-bearing polymer-drug conjugate analogue **1.3** (Figure 1.7, see Section 4.3.2.8 for a further discussion and investigation of this reaction). The reaction was followed by FTIR spectroscopy, with an observed quantifiable loss of the activated ester carbonyl signal at 1735 cm^{-1}.



1.3

Figure 1.7: The reported synthesis of HPMA copolymer **1.3** from pMAOS.

This synthetic route has two important advantages over the former. Firstly, the activated polymeric precursor, pMAOS, can be synthesised with a low PD, and, secondly, a single polymeric precursor can in theory be used to synthesise any number of polymer therapeutics incorporating different drugs, biolinkers and (if desired) targeting residues, and all with variable levels of loading. In short, a range of polymeric constructs can be synthesised from only the one precursor, which can be easily made in high quality.

While to date there have been no further reported syntheses of polymer-drug conjugates utilising this type of chemistry, the methodology was employed by the same group in the synthesis of a range of poly(methacrylamide) copolymers.[61] Furthermore, a 2004 Monge and Haddleton paper reported an optimised synthesis of pMAOS using controlled radical polymerisation protocols, as well as the subsequent aminolysis of the product with benzylamine to give the benzylamide homopolymer product.[62]

This pMAOS-based methodology was further investigated in the work described in this thesis (see chapter 4), with an aim to synthesise conjugates of type **1.1** bearing anticancer drugs. Specifically, variolins, discussed below, were employed as the cytotoxic compounds.

1.3 The Variolins

1.3.1 Discovery

Two related papers published in 1994 by Perry *et al.*[63] and Trimurtulu *et al.*[64] reported on the discovery of a new class of marine alkaloids, the variolins, from the Antarctic sponge *Kirkpatrickia varialosa* (Figure 1.8). These compounds all share a novel tricyclic pyridopyrrolopyrimidine aromatic system otherwise unseen in natural products.

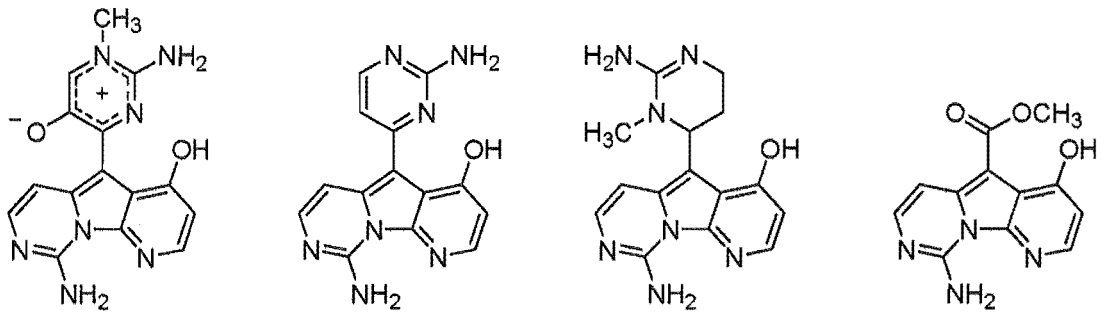


Figure 1.8: The four variolin structures reported in 1994, a) variolin A, b) variolin B, c) *N*(3')-methyl tetrahydrovariolin B, and d) variolin D (a suspected artefact).

Of the three natural products and single suspected artefact (variolin D) that were discovered, one in particular, variolin B (Figure 1.8b), showed pronounced anticancer cytotoxicity *in vitro* against the murine P388 leukaemia cell line, with an IC_{50} of 210 ng/mL, or 0.72 μM. An X-ray crystal structure showed the pendant pyrimidine ring of variolin B to be tilted out of the plane by 24°, with the ^1H NMR spectrum confirming that the hydroxyl proton was strongly hydrogen bonded.[63]

1.3.2 Mode of action of variolin B

Variolin B exerts its cytotoxicity towards cancer cells through the rapid induction of apoptosis, specifically, via the intrinsic, or mitochondrial, pathway.[65] Variolin B was initially suspected to be an intercalator due to structural similarities to other known intercalators.[66] However, it is now known to be a cyclin-dependent kinase (CDK) inhibitor.[65] The IC_{50} value of variolin B towards the human CDK1 and CDK5 enzymes *in vitro* was shown to be 60 nM and 90 nM, respectively.[67] Dysfunction (specifically, overexpression) of the CDK pathway, a crucially important pathway in the regulation of the cell cycle, is seen in most, if not all, human tumours.[68] Apoptosis-inducing CDK inhibitors have therefore been the focus of extensive interest in cancer therapy, with several such inhibitors currently in clinical trials.[68-70] While structurally diverse, all other known CDK inhibitors are flat, hydrophobic heterocyclic compounds of molecular weight less than 600 $gmol^{-1}$, which all compete with ATP for binding in the kinase ATP-binding site.[69]

Furthermore, variolin B is cytotoxic towards multiple-drug resistant (MDR) cells, and its cytotoxicity appears to be independent of the p53 status of the cells.[65] The protein p53 is a transcription factor of central importance in the regulation of cell proliferation, differentiation, DNA repair and apoptosis.[71] Virtually all cancer cells possess a defective p53 system, and it is proposed that the observed chemoresistance of many tumours is due to this defective system (especially with respect to cytostatic and apoptosis-inducing drugs).[72]

The p53-independence of the mode of action of variolin B, along with the observation that CDK inhibitors will produce synergistic effects when administered in conjunction with conventional anti-tumour agents,[70] makes variolin B (and deoxyvariolin B, discussed in Section 1.3.3.1, which has a very similar biological activity profile) a very exciting lead compound in the treatment of cancer.

1.3.3 Synthesis

Given the inaccessibility and world heritage status of Antarctica, there was significant interest in the development of a synthesis of variolin B to provide material (including non-natural analogues) for further investigations.

The synthetic protocols employed in the work described in this thesis are those developed by Anderson and Morris (see Sections 2.2 and 1.3.3.1). Before outlining these protocols, however, a brief overview of the other reported syntheses from three separate groups will be given.

Fresneda, Molina and coworkers were the first to report a synthesis of the pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine variolin core, **1.4** (Figure 1.9).[73] The synthesis was extended to give variolin B (shown retrosynthetically in Figure 1.9) in 13 steps with 6.5% overall yield.[74,75]

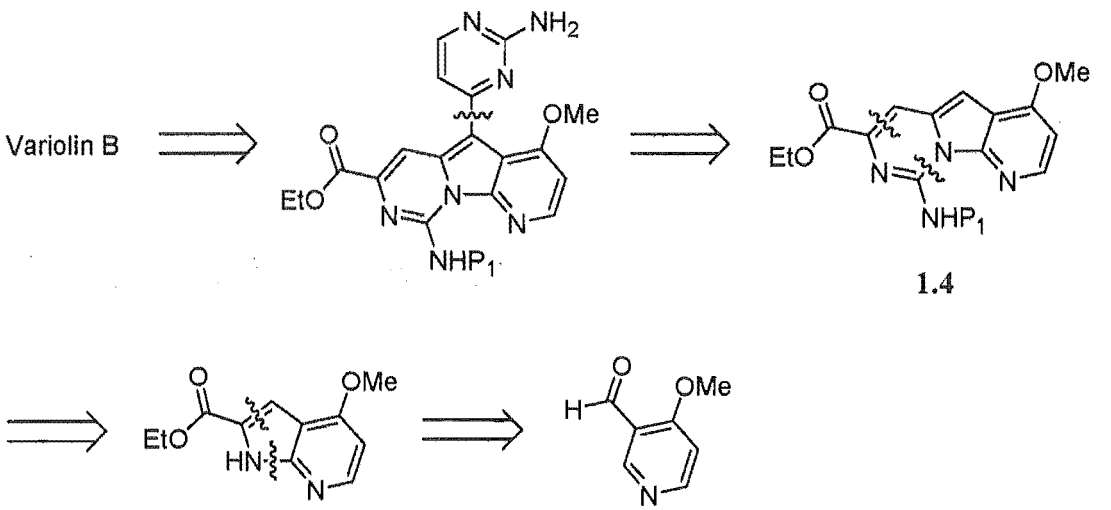


Figure 1.9: A retrosynthetic analysis of Fresneda and Molina's route to variolin B.

Vaquero and colleagues also developed a synthesis of the variolin core, **1.5** (Figure 1.10, R = CO_2Me).[76,77] Having altered their synthetic approach from an earlier proposed route,[78] the researchers were served by serendipity in the development of a two step procedure with 49% overall yield. Their synthesis is shown retrosynthetically in Figure 1.10.

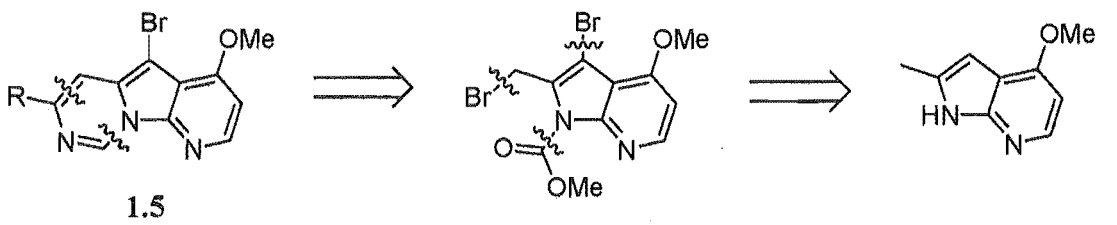


Figure 1.10: Retrosynthetic analysis of the route developed by Vaquero *et al.*

The analogous deoxy core (deoxy **1.5**, R = CO_2Me) was then synthesised by a similar route in two steps with 53% overall yield. This species was arylated at the C(5) position with the appropriate 2-thiomethyl pyrimidine stannane in a cross coupling reaction in 63% yield. The methyl ester was also removed from deoxy **1.5** by ester hydrolysis under basic conditions followed by decarboxylation in diphenyl ether at 250 °C to give deoxy **1.5** (R = H) with 30% yield over two steps.

Finally, Alvarez, Fernández and Joule worked on the following retrosynthetic pathway (Figure 1.11). In 1999 they reported on the synthesis of fragments of the variolin core structure,[79,80] proving the validity of the proposed synthetic pathway. In 2001 they reported the synthesis of the non-natural analogue deoxyvariolin B (DVB, Figure 1.13) via the proposed pathway.[81] In 2002 they reported a synthesis of a further key variolin fragment,[82] then in 2003, along with other researchers based in Spain, they reported the total synthesis of variolin B in ten steps and 1.4% overall yield.[83,84] DVB was also synthesised using a similar strategy in ten steps and 4.1% overall yield.[84]

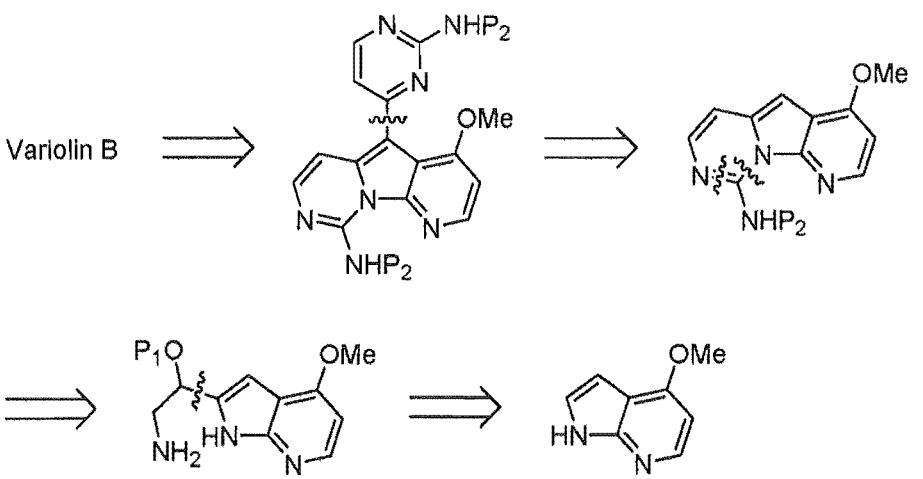


Figure 1.11: The Alvarez, Fernández and Joule retrosynthetic pathway to variolin B.

1.3.3.1 The Anderson/Morris variolin B and deoxyvariolin B syntheses

In 2001 Dr Regan Anderson, a doctorate student at the time supervised by Dr Jonathan Morris, completed the first total syntheses of both variolin B and the non-natural analogue DVB.[85-88] The synthetic strategy was based on the insight that variolin B possesses a hidden element of symmetry. The initial synthetic route that was devised (shown retrosynthetically in Figure 1.13, and discussed below with respect to the deoxy series) gave favourable results when applied to

the deoxy series, but unsatisfactory results when applied to the methoxy series. The key shortcoming was an inefficient synthesis of the triaryl methanol species. For this reason, the retrosynthetic pathway shown in Figure 1.12 was developed.

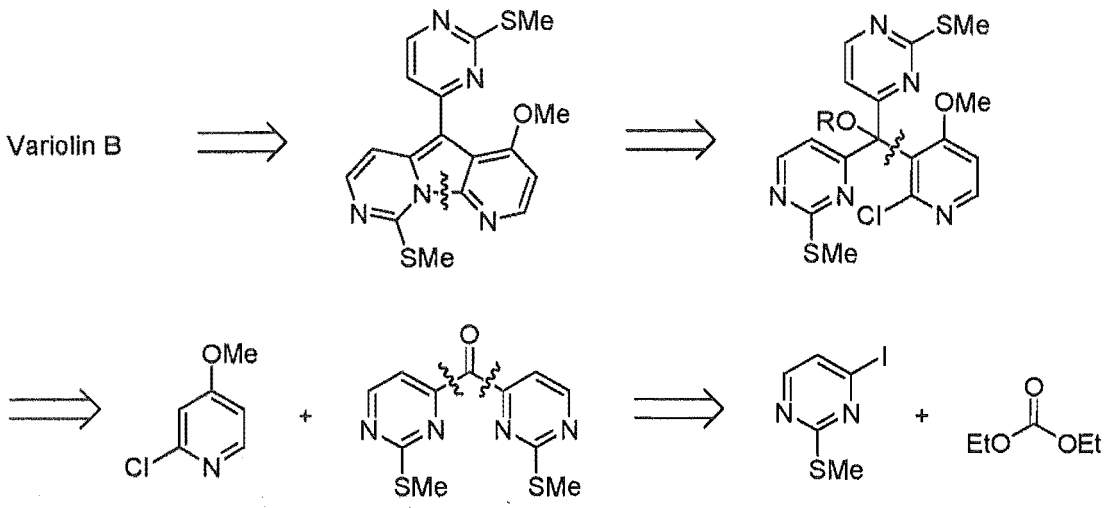


Figure 1.12: A retrosynthetic analysis of the Anderson/Morris synthesis of variolin B.

This synthetic route involves the synthesis of a symmetrical ketone from the reaction of diethyl carbonate with the lithio species formed *in situ* from 4-iodo-2-thiomethylpyrimidine. This ketone is then arylated by the lithio species formed *in situ* from 2-chloro-4-methoxypyridine to give the triaryl methanol. Attempted deoxygenation of this compound using triethyl silane and TFA led to concomitant ring-closing to give the bis-thiomethyl core structure. This key reaction step was subsequently optimised by decreasing the amount of TFA used, and was also found to proceed more efficiently from the triaryl acetate. Introduction of the amino groups onto the pyrimidine rings of the core structure gave a triprotected compound that could be deprotected to give variolin B in 8 steps and 15% overall yield.

As was mentioned above, though not useful for the synthesis of the triaryl methanol required for the synthesis of variolin B, the synthetic route that was first pursued (Figure 1.13) gave good results in the deoxy series (which is discussed in more detail in Section 2.2). The synthesis involves the biarylation of 2-chloro-nicotinoyl chloride with the 4-lithio-2-thiomethylpyrimidine nucleophile (formed *in situ* from the iodide) to give a triaryl methanol species. The triaryl methanol was taken through to give the variolin core structure in one step (unlike in the methoxy series, the triaryl acetate was not investigated in the deoxy series). As for the methoxy series, introduction of the amino groups onto the pyrimidine rings was achieved by an

m-chloroperbenzoic acid oxidation of the thiomethyls to give the bis sulfone intermediate, followed by displacement of both sulfone moieties by *p*-methoxybenzylamine. The *p*-methoxybenzylamine groups of the resulting intermediate were removed by stirring with neat triflic acid to give DVB. DVB was therefore synthesised in six steps with 5.2% overall yield. Each of these synthetic steps is dealt with in more detail in Sections 2.2.1, 2.4.1 and 2.5.1.

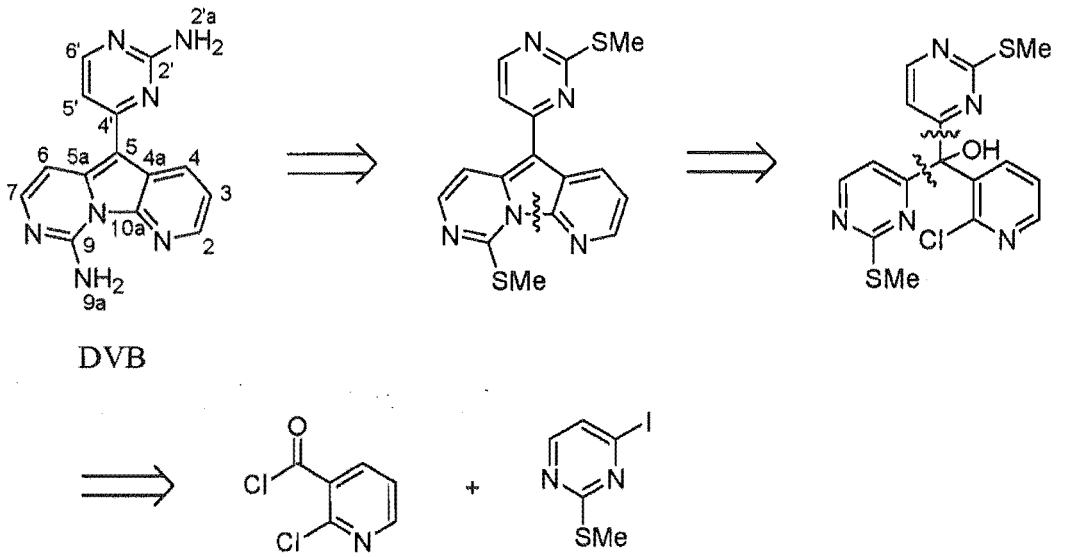


Figure 1.13: A retrosynthetic analysis of the Anderson/Morris synthesis of DVB.

DVB is slightly more bioactive than variolin B, with an IC_{50} of 0.47 μM against the murine leukaemia P388 cell line. DVB has the further advantage of being more synthetically accessible than variolin B, and so the DVB core structure became the focus of the work described in this thesis.

1.3.3.2 Analogue syntheses

Anderson and Morris also devised a series of synthetic routes to variolin analogues, with the potential to selectively functionalise at the C(2') and/or C(9) positions (Figure 1.14), and employed these protocols in the synthesis of a range of non-natural analogues. The analogues that were synthesised, along with their bioactivities, are given in Section 3.2.2.

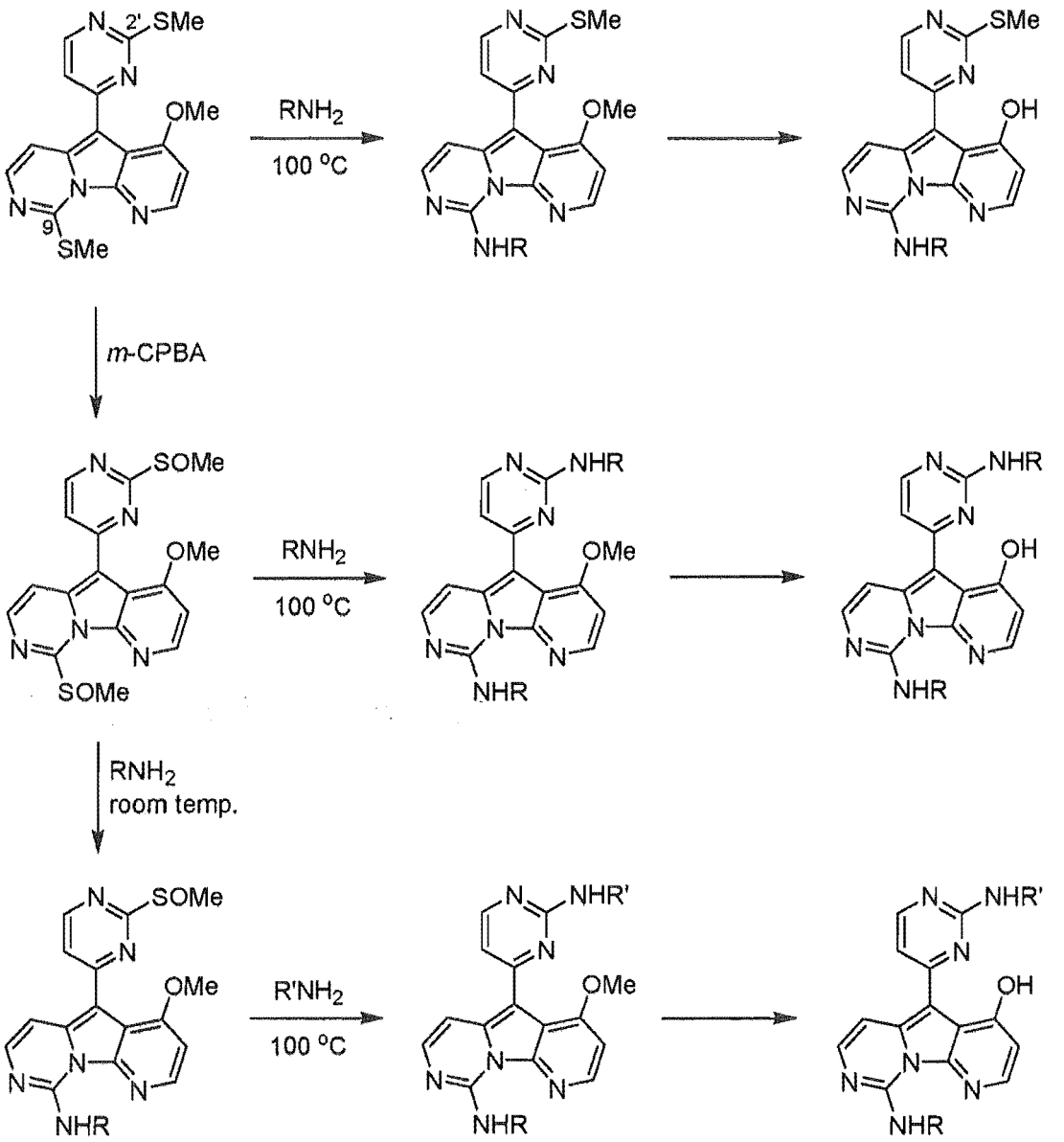


Figure 1.14: The synthetic routes devised by Anderson and Morris in the synthesis of non-natural variolin analogues.

The brief structure-activity relationship (SAR) investigation carried out by Anderson and Morris was extended to the deoxy series as part of the work described in this thesis, with an aim to determine the most suitable analogues for further development into polymer therapeutics (see Section 3.2.3).

It should be noted that this SAR work was carried out as part of a much larger SAR effort coordinated by Pharma Mar, SA. Their work will be discussed in more detail in Section 3.1.

1.3.4 Reasons for developing DVB into a polymer therapeutic

DVB is a compound in the early stages of development as an anticancer therapeutic. It is slightly more bioactive and more synthetically accessible than the parent natural product variolin B. It possesses appreciable anticancer activity *in vitro*, and preliminary investigations using animal models have begun. One of the most significant shortcomings of this promising compound, however, is its low aqueous solubility. The use of solubilising formulations has thus far been met with only partial success.[89] The pharmaceutical utility of DVB in its unmodified form will therefore almost certainly be limited by this poor solubility. Conjugation with a water soluble polymer, such as pHPMA, will greatly increase the water solubility of DVB. The further advantages associated with macromolecular prodrugs, namely improved pharmacokinetics and pharmacological profiles, have been discussed in detail in 1.2.2.

1.4 Aims

At the beginning of the work described in this thesis, the aims of the work were defined as follows:

1) to further investigate and optimise the synthesis of DVB;

2) to extend the brief SAR investigation involving the methoxy and hydroxyvariolin series to the deoxy series;

3) to select the most suitable analogues for further development into polymer-drug conjugates, and acylate these analogues with the GFLG tetrapeptide biolinker; and,

4) to use any tetrapeptide-variolin conjugates successfully synthesised in the synthesis of polymer-drug conjugates from pMAOS.

Chapter 2 outlines the optimisation of the synthesis of DVB, as well as the attempted synthesis of a DVB-tetrapeptide conjugate. Attempts were also made at synthesising a variolin analogue-tetrapeptide conjugate, with the variolin being bound through the N(9a) position. Some interesting chemistry at the N(9a) position was uncovered during this work.

Chapter 3 outlines the synthesis of a small family of DVB analogues, with a tetrapeptide conjugate being synthesised from the most suitable of the analogues.

Finally, Chapter 4 outlines the discovery and characterisation of some unforeseen side reactions encountered in the use of pMAOS as an activated polymeric precursor in the synthesis of polymer-drug conjugates.

CHAPTER 2

ACYLATION OF THE VARIOLIN CORE

2.1 Introduction

As was discussed in Chapter 1, DVB was to be incorporated in a polymer therapeutic via a biodegradable tetrapeptide biolinker. It was therefore necessary to acylate DVB with the *N*-protected tetrapeptide Fmoc-glycyl-L-phenylalanyl-L-leucyl-glycine.

DVB has two potential points of attachment – the N(9a) and N(2'a) positions. This chapter deals first with the synthesis of the variolin core, then with attempts to selectively acylate at each of these positions.

2.2 Characterisation of Compounds

All new compounds synthesised as part of the work described in this and in following chapters were rigorously characterised using a range of techniques (compounds that had previously been reported were generally characterised by ^{1}H NMR and melting point analysis alone). NMR experiments that were routinely performed included 1D ^{1}H and ^{13}C experiments, and the 2D COSY, HSQC-DEPT and CIGAR experiments. The type of information that is afforded by these 2D experiments is shown below in Figure 2.1.

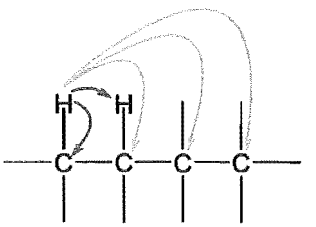


Figure 2.1: The specific types of bond connectivities detected by COSY (red), HSQC (blue) and CIGAR (green) 2D NMR experiments.

COSY experiments indicate pairs of adjacent protons. HSQC-DEPT experiments indicate the direct connectivity of proton(s) to the carbon to which they are bonded, as well as establishing if a given proton signal is a methylene or a methine/methyl group. CIGAR experiments give correlations between protons and carbon atoms separated by two, three or four bonds (with the latter being less common).

All novel compounds synthesised as part of the work described in this thesis were unambiguously characterised by a combination of 1D and 2D NMR techniques. Around half of the most important previously reported compounds were also characterised in this manner. In all but one example (which is discussed in Section 2.4.3.1), these three techniques allowed unambiguous assignment of all ^{1}H and ^{13}C signals in the respective 1D NMR spectra. The 2D NMR techniques were particularly useful in the structure elucidation of copolymers **4.3** and **4.6**, discussed in detail in Sections 4.3.2.1 and 4.3.2.6, respectively.

In addition to characterisation by 2D NMR spectroscopy, all novel compounds synthesised as part of this work were routinely analysed by high-resolution mass spectrometry (HRMS) using electrospray ionisation (ESI), a technique which employs a 'soft' form of ionisation that leads to minimal decomposition of the parent ion, with detection being carried out in the positive mode. HRMS analyses were performed in quadruplicate, with a requirement being that the root-mean-square (rms) of the deviation from the expected value for the four runs be less than 5 ppm for a result to be considered confirmative.

Other routinely used characterisation techniques included melting point determination of crystalline solids and FTIR spectroscopy. HPLC and LCMS were also employed at times to lend support to proposed structures and/or the purity of samples.

2.3 Synthesis of the Deoxyvariolin Core

The Anderson and Morris route to the deoxyvariolin core structure is shown below in Figure 2.2. The final step (an ionic hydrogenation[90] involving ring-closing and reduction of the triaryl species in one reaction step) was initially performed on the triaryl alcohol **2.4**,[86] though further work on the analogous methoxy system had indicated that this reaction would proceed in better yield from the triaryl acetate, **2.5**.[87] DVB was synthesised in 6 steps in 5.2% overall yield.

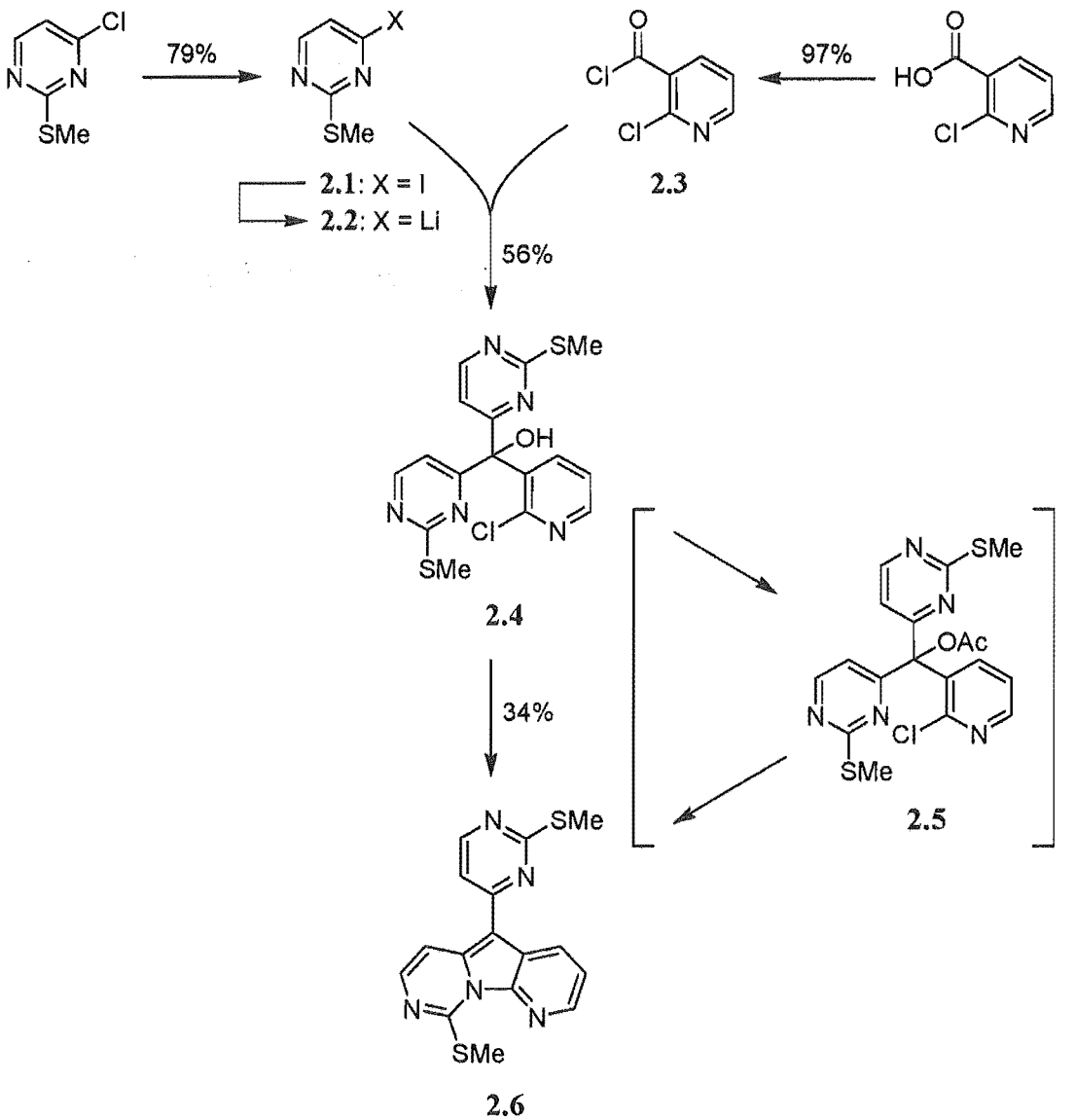


Figure 2.2: The Morris and Anderson synthetic route to the deoxyvariolin core.

Given the need of a ready supply of deoxyvariolin material, it was decided that these synthetic protocols would be more closely investigated and hopefully improved.

2.3.1 Replication of the existing protocols

The protocols for the synthesis of the iodopyrimidine **2.1** and pyridine acid chloride **2.3** were replicated to give the desired products in 80% and 99% yield, respectively. These two reagents were then used in the synthesis of triaryl alcohol **2.4** using the protocols developed by Anderson and Morris. These protocols involved the addition of **2.3** to the lithio species **2.2**, which is formed *in situ* from **2.1**. Species **2.2** is relatively unstable and prone to side reactions. The exothermic nature of the addition reaction further complicates this troublesome reactivity in larger scale syntheses. A specialized piece of glassware was therefore employed – a reactor with a coiled side arm for the addition of reagents.[87] During the reaction the reactor is almost completely immersed in a -95 °C ($MeOH/N_2$) cold bath, so that reagents that are added via the side arm are cooled before reaching the reaction solution.

The protocols specify the careful addition of *n*-BuLi at 0.5 mL/min to a vigorously stirred solution of iodide **2.1**, stirring the reaction solution for 20 minutes to allow the complete formation of **2.2**, then the careful addition of acid chloride **2.3** at a similar rate followed by stirring for a further 3-4 h. During the course of the reaction the temperature should remain as close to -95 °C as possible, and of course, must be kept rigorously dry. This is a difficult reaction, but yields of ~55% can be expected if carried out properly.

The first attempt at this reaction gave only a 26% yield of triaryl alcohol **2.4**. The exact reason for the low yield was unknown, but was most likely due to flawed experimental technique. It was decided to convert this material to triaryl acetate **2.5**, with an aim to take the acetate through to the ionic hydrogenation step in the hope that the observed improvement in yields for the methoxy system would hold true in the deoxy series.

Triaryl alcohol **2.4** was therefore acetylated with acetic anhydride to give triaryl acetate **2.5** in 92% yield. **2.5** was then subjected to the ionic hydrogenation protocols, involving triethyl silane and TFA, to give core structure **2.6** in 69% yield, thus confirming that the triaryl acetate gives much better yields than the triaryl alcohol in both the deoxy as well as the methoxy system.

The synthetic protocols described above for the synthesis of **2.4** were repeated, differing only in that an acetyl chloride quench was used in place of the methanol quench upon completion of the reaction (in analogy to the protocols developed for the methoxy system), to give triaryl acetate **2.5** in 60% yield.

2.3.2 Arylation of Weinreb's amide **2.8**

Despite the good yield in this reaction, it was still considered desirable to investigate other synthetic approaches employing a more robust triaryl acetate synthetic step. Therefore, a similar synthetic route reported by Remuinan *et al.* was investigated.[91] This procedure involves the synthesis of the Weinreb's amide **2.8** from acid chloride **2.3**, which is then reacted with the Grignard species **2.7**, formed *in situ* from iodide **2.1**, to give ketone **2.9**. **2.9** is then reacted in a subsequent step with **2.7** in a reaction quenched with acetyl chloride to give triaryl acetate **2.5** in a reported 52% yield over 3 steps (Figure 2.3).

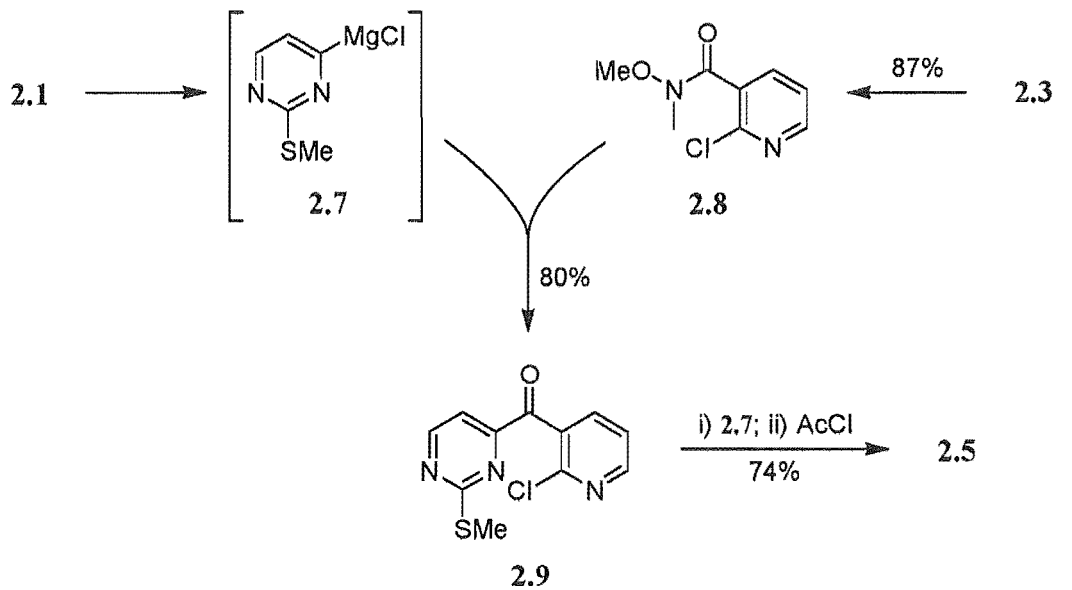
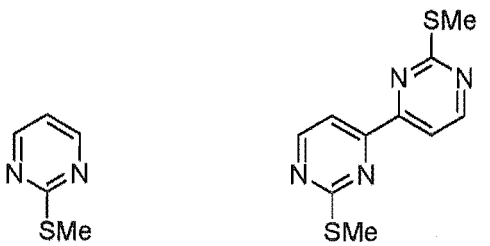


Figure 2.3: The Remuinan *et al.* synthetic route to triaryl acetate **2.5**.

Thus, Weinreb's amide **2.8** was synthesised from acid chloride **2.3** in 93% yield. However, the reaction of **2.8** with **2.7** (formed *in situ* from **2.1**) did not proceed as cleanly as was hoped. The literature protocols specified that Grignard reagent **2.7** was formed from **2.1** at 0 °C then transferred via cannula into a solution of **2.8**. These protocols were eventually modified slightly,

so that **2.7** was formed at -15 °C and into this solution was then carefully transferred via cannula a solution of **2.8** to give ketone **2.9** in 75% yield. The subsequent step, involving the arylation of ketone **2.9** by **2.7**, gave mixtures of compounds, of which the desired product **2.5** was only a small component (as determined by ^{1}H NMR spectroscopy). The crude reaction mixtures typically contained 10-20% unreacted starting material **2.9** as well as a large amount of the two compounds **2.10** and, to a lesser extent, **2.11** (Figure 2.4). The desired product, **2.5**, was isolated in 14% yield.



2.10 **2.11**

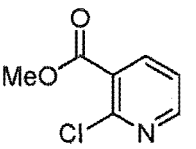
Figure 2.4: Side products **2.10** and **2.11**.

The presence of both **2.9** and **2.11** in the crude reaction mixtures indicated that the nucleophile was consumed by side reactions before the reaction was able to go to completion. This is despite using a 2.5 molar excess of the nucleophile. In the synthesis of **2.9** from **2.8**, only a 1.25 molar excess of **2.7** was required, indicating that the formation of **2.7** from **2.1** was an efficient reaction. The difference in the success of the two reactions therefore most likely reflects the higher degree of steric hindrance in the ketone compared with the Weinreb's amide. It is not known why the literature account encountered more success than was seen in this work.

In order to address the problem of the relatively low reactivity of ketone **2.9**, the stonger lithio nucleophile **2.2** was investigated. The literature reports the use of Barbier protocols in the arylation of **2.9** by **2.2** (specifically, the addition of *n*-BuLi to a cooled solution of both **2.9** and **2.1** at -78 °C) to give **2.5** in 61% yield.[91] These protocols were therefore repeated to give **2.5** in 59% yield.

2.3.3 Arylation of methyl ester **2.12**

Work being carried out in parallel to the Weinreb's amide investigation involved the arylation of methyl ester **2.12** (Figure 2.5) with either **2.2** or **2.7**.

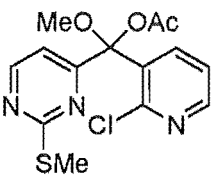


2.12

Figure 2.5: Pyridine methyl ester **2.12**.

Ester **2.12** was synthesised from **2.3** in 85% yield. Arylation of **2.12** with three molar equivalents of **2.7** gave a messy crude reaction mixture (as determined by ^{1}H NMR spectroscopy) comprised of the **2.12** starting material, ketone **2.9**, side product **2.11** and, to a lesser extent, product **2.5**. Attempts were not made to purify the product from this mixture.

It appeared that, as in the arylation of **2.9**, the stronger lithio nucleophile **2.2** was required. Thus, **2.12** was reacted with three molar equivalents of **2.2** using Barbier protocols and the reaction quenched with acetyl chloride. Rather than the expected product **2.5**, what was instead isolated from the reaction mixture, in 90% yield, was the quaternary species **2.13** (Figure 2.6).



2.13

Figure 2.6: Quaternary species **2.13**.

It is postulated that **2.13** forms due to the stabilisation of the quaternary intermediate oxyanion through coordination by the lithium cation (Figure 2.7).

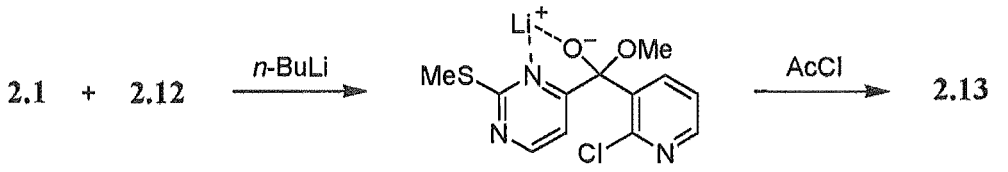


Figure 2.7: Proposed mechanism of formation of **2.13**.

Treatment of **2.13** with aqueous NaOH caused it to collapse to ketone **2.9**. Thus, when a crude reaction mixture from the reaction shown in Figure 2.7 was treated with 1 M NaOH at room temperature for 2 h and the crude reaction mixture analysed by ^{1}H NMR spectroscopy, ketone **2.9** was seen by to be the only significant nicotinoyl-containing compound. Unfortunately, purification of **2.9** was hindered by the coelution of side product **2.10**, which forms from unreacted **2.2**. It is anticipated that a lower stoichiometry of **2.2** in the above reaction (1.2 equivalents, for example) would be sufficient for the reaction to go to completion and would avoid the problems of contamination by **2.10**.

2.3.4 Barbier protocols with acid chloride 2.3

With the observed successes seen with the application of Barbier protocols in the synthesis of triaryl acetate **2.5** from **2.9**, it was decided to employ these protocols in the synthesis of **2.5** (and, for comparative purposes, triaryl alcohol **2.4**) from acid chloride **2.3** and iodide **2.1** (Figure 2.8).

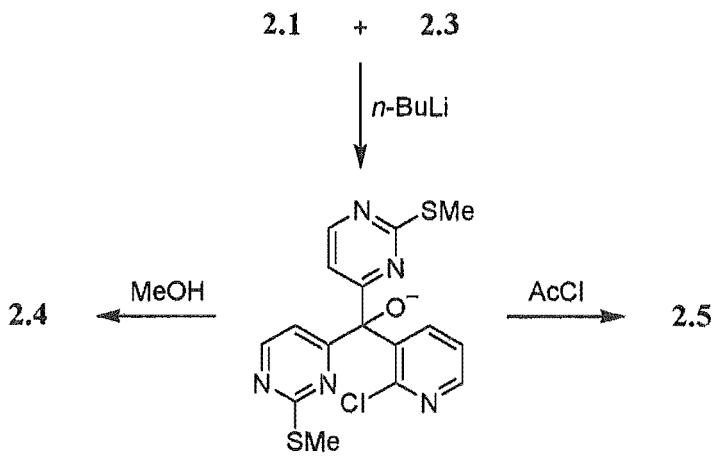
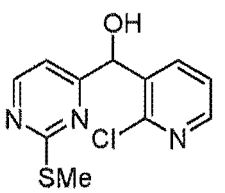


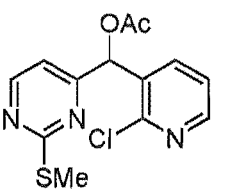
Figure 2.8: Syntheses of **2.4** and **2.5** from **2.1** and **2.3** under Barbier lithiation conditions.

Initial investigations gave promising results, and three small modifications were made: firstly, it was best to transfer a solution of the iodide to a cooled solution of the acid chloride immediately prior to addition of *n*-BuLi, rather than letting the two reagents sit together for any length of time (during which time side reactions, leading to the formation of what appeared to be I_2, were seen to occur); secondly, a reaction temperature of -96 °C gave superior yields than one of -78 °C; and, thirdly, 2.2 molar equivalents of the nucleophile were sufficient for the reaction to proceed to completion.

The protocols employing the methanol quench gave an inseparable mixture of triaryl alcohol **2.4** with a small amount of the biaryl alcohol **2.14** (Figure 2.9). This mixture was refluxed in benzene with MnO_2 to give a mixture of ketone **2.9** and **2.4**, the latter of which was then easily purified in 40% yield. When the acetyl chloride quench was used, triaryl acetate **2.5** was obtained in 50% yield. In some instances an inseparable mixture of **2.5** and biaryl acetate **2.15** (Figure 2.9) was obtained; this material, however, could be taken through to the next synthetic step to give core structure **2.6**, which could then be easily purified away from the contaminant **2.15** in good yield. The formation of **2.14** and **2.15** under similar conditions have been reported by Anderson and Remuinan *et al.*, respectively.[87,91]



2.14



2.15

Figure 2.9: Contaminants **2.14** and **2.15**.

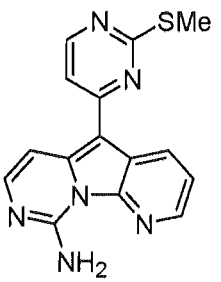
While the yields were reasonable, especially considering the more simple reaction protocols and much lower nucleophile stoichiometry, the yields were still not as good as expected based on analysis of the crude reaction mixtures by ^{1}H NMR spectroscopy, which indicated yields of around 80%. Furthermore, purifications that involved several chromatographic steps consistently gave lower yields, suggested that material was being lost during the chromatography. Using DIOL in the place of silica gave no better results. For this reason a crude reaction mixture was taken directly through to the core structure synthetic step to give **2.6** in 50% yield over 2 steps. Given that the ring-closing reaction proceeds in about 70% yield from

a clean sample of **2.5**, this yield of 50% over 2 steps confirms the high yield suggested by the ^{1}H NMR results for the synthesis of **2.5** and supports the assertion that material was being lost during chromatography.

Thus, core structure **2.6** was synthesised over 3 steps (with only 1 chromatographic step, and one step requiring no purification at all) from commercially available starting materials in 40% overall yield.

2.4 Acylation of N(9a)

With core structure **2.6** in hand, and ready access to more if needed, attention could now be turned towards the synthesis of acylated analogues of DVB. Rather than investigating the acylation of DVB immediately, it was considered desirable to first synthesise analogue **2.16** (Figure 2.10) to investigate acylations exclusively at the N(9a) position.



2.16

Figure 2.10: Analogue **2.16**.

2.4.1 Synthesis of **2.16**

Intermediate **2.17** was synthesised in near-quantitative yield via the synthetic route shown in Figure 2.11, a route developed by Anderson and Morris during investigations involving the methoxy system.[87] Deprotection of the *p*-methoxybenzylamine (PMB) group from **2.17** with neat triflic acid to give analogue **2.16** proceeded without complications in good yield.

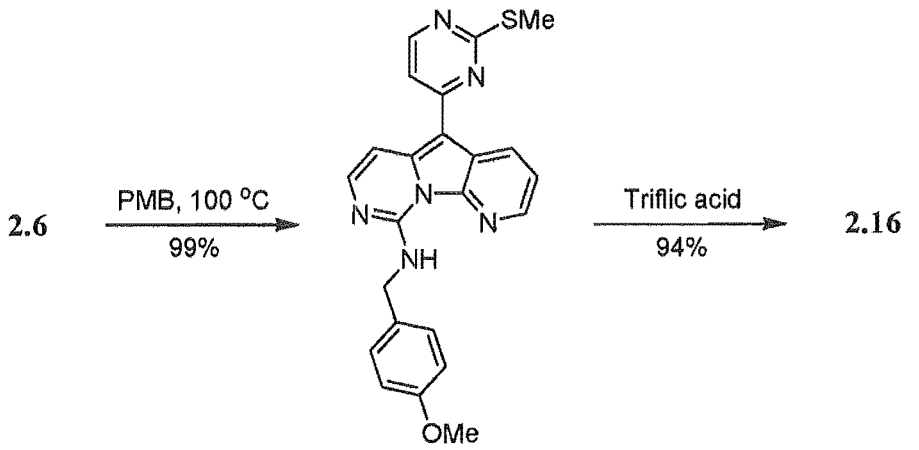


Figure 2.11: The synthetic route to analogue **2.16** from core structure **2.6**.

Analogue **2.16** posessed an IC_{50} of 3.9 μM against the P388 cell line. Although this was approximately an order of magnitude lower than the bioactivity of DVB (IC_{50} = 0.47 μM) against this particular cell line, it was still considered a worthwhile candidate for further development into a polymer therapeutic (see Section 3.2.3.4 for a discussion of *in vitro* bioactivity in the context of this work). It was also a logical choice of analogue for the investigation of acylations at the N(9a) position.

2.4.2 Tetrapeptide biolinker synthesis

It was necessary to synthesise the tetrapeptide biolinker - FmocGFLG - to be used as the electrophile in the acylation of analogue **2.16**. Given that the cytotoxins being employed in this work were all of synthetic origin, it was desirable to have ready access to comparable amounts of the tetrapeptide. Several synthetic routes were therefore investigated.

2.4.2.1 Solid-phase protocols

Solid-phase protocols employing Fmoc-protected amino acids[92] and Wang-resin[93] had previously been applied in the synthesis of FmocGFLG.[94] HBTU couplings were employed, with Fmoc-deprotection being carried out using piperidine. These protocols were successfully repeated to give the tetrapeptide in 38% overall yield, but it was soon realised that, unlike the natural product-based projects for which this synthesis had been developed, the work being carried out

here required relatively large amounts of this compound. It was therefore decided to develop solution-phase protocols for the simple (and less expensive) gram-scale synthesis of the biolinker.

2.4.2.2 Solution phase protocols employing Fmoc-amino acid chlorides

Carpino and coworkers have reported the rapid and convenient synthesis of oligopeptides (up to 22 residues) in the solution phase using Fmoc-protected amino acid chlorides.[95-98] A chloroform solution of the carboxyl-protected peptide nucleophile with a small excess of the Fmoc-protected amino acid chloride is vigorously stirred with a 5% aqueous Na_2CO_3 solution. The organic fraction (containing all the peptidic material) is then isolated from this biphasic mixture and treated with a diamine base, which serves to both cleave the Fmoc goups and react with any remaining acid chloride. The mixture is then extracted with pH = 5.5 phosphate buffer to remove the basic fulvene adducts and conjugates, while leaving the less basic free amine growing peptide chain in the organic phase for the next coupling cycle. Importantly, racemization was not observed during the application of these protocols.[95] Figure 2.12 shows one of these coupling/deprotection cycles, employing 4-aminomethyl piperidine (AMP) as deprotecting agent and nucleophilic scavenger (nucleophilic attack may involve either of the two nitrogens, hence the ambiguous structures).

The Fmoc-amino acid chlorides were easily synthesised from their respective amino acids by reaction with thionyl chloride,[95] with the exception of Fmoc-L-leucine acid chloride, which was successfully synthesised but used as a thick gum, as repeated attempts at recrystallisation were unsuccessful. The tetrapeptide was then synthesised via the above protocols, albeit in only low overall yield - a yield of 28% (24% if the glycine benzyl ester synthetic step is included) was achieved once, with yields of 10-15% being typical.

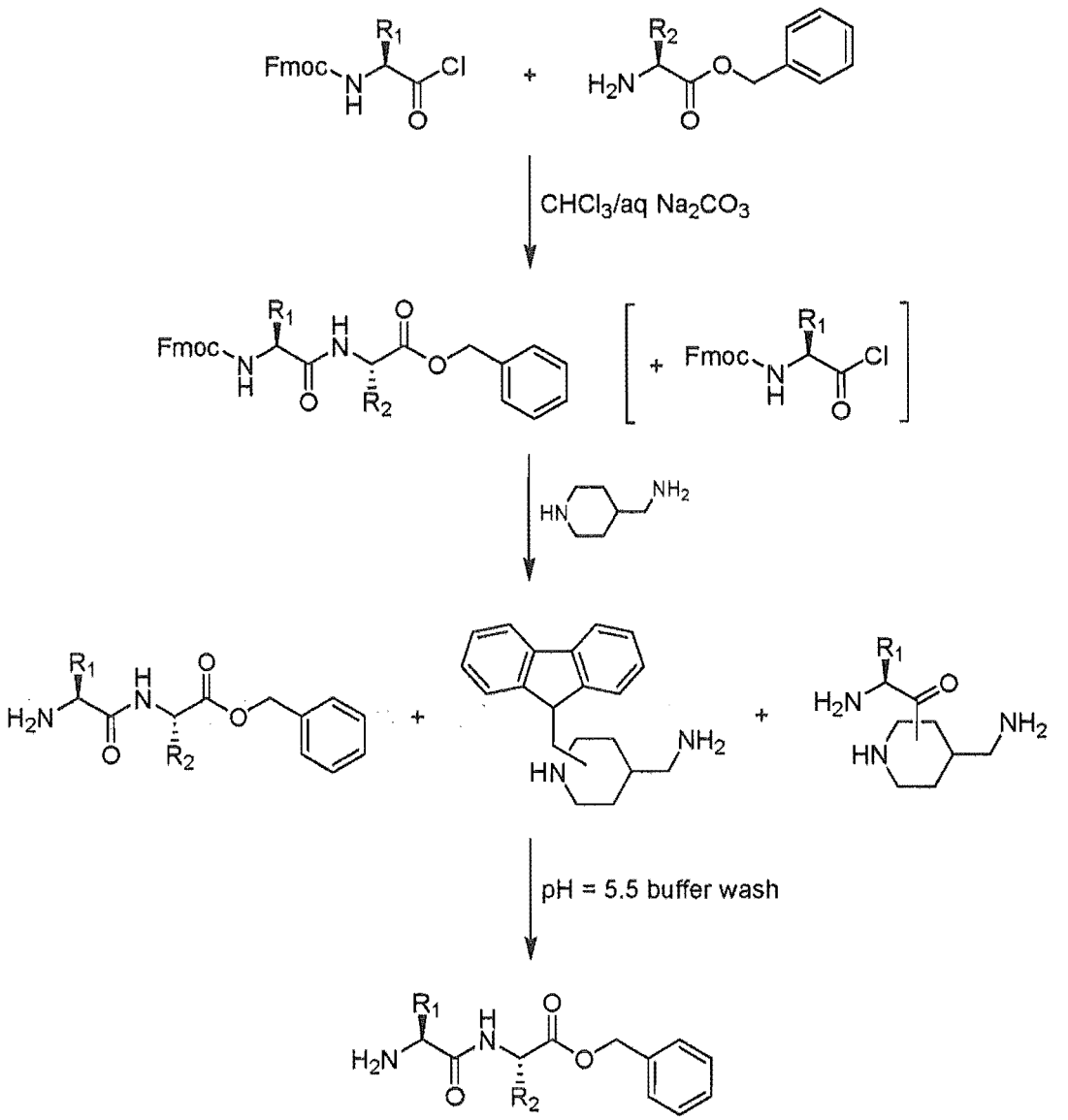


Figure 2.12: One cycle of a solution phase synthesis of oligopeptides using Fmoc-protected amino acid chlorides.

A closer look at the first two steps revealed the reason for the poor yield. Firstly, it had been observed during ^{1}H NMR spectroscopic analysis of the acid chlorides that Fmoc-L-leucine was much more electrophilic than the other two amino acid chlorides (in contrast to Fmoc-glycine and Fmoc-L-phenylalanine acid chlorides, relatively large amounts of the free acid of Fmoc-L-leucine were observed in NMR spectra of this acid chloride due to traces of water in the $CDCl_3$

NMR solvent).* Hydrolysis during the coupling reaction involving Fmoc-L-leucine acid chloride therefore represented a significant loss of material. Secondly, diketopiperazine formation following deprotection of the dipeptide (Figure 2.13) was also thought to be occurring, though efforts were not made to purify the diketopiperazine.

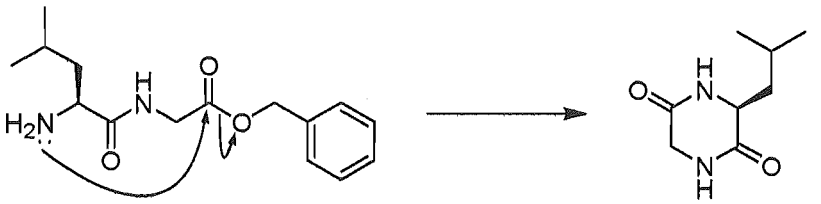


Figure 2.13: Presumed diketopiperazine formation from LGOBn.

Due to this unsatisfactory first coupling/deprotection cycle, giving 50% yield at best, it was decided to use more conventional solution-phase protocols in the synthesis of the tetrapeptide.

2.4.2.3 Conventional solution-phase protocols

The tetrapeptide was synthesised in the *C-N* direction, with the *C*-terminal glycine residue being protected with a benzyl ester group. Fmoc-protected amino acids were used. The first coupling, between the benzenesulfonate salt of glycine benzyl ester and Fmoc-L-leucine, was carried out with DCC/HOBT, with the dipeptide being easily separated from the DCU side product by silica chromatography. The Fmoc group was cleaved from FmocLGOBn by stirring with 1:1 AMP/THF for five minutes and the deprotection adduct removed by washing with pH = 5.5 phosphate buffer (these manipulations were carried out as quickly as possible in an attempt to minimise diketopiperazine formation). To the reaction solution was then immediately added Fmoc-L-phenylalanine acid chloride and 5% aqueous Na_2CO_3 solution, the biphasic solution stirred vigorously at room temperature, and the product purified by silica chromatography. AMP-deprotection protocols were again employed in the deprotection of the Fmoc group, an EDCI/HOBT coupling used in the reaction between the resulting tripeptide and Fmoc-glycine, and the tetrapeptide benzyl ester purified on silica. The benzyl ester was cleaved by palladium-

* Synthesis of leucine methyl ester from the acid chloride with dry methanol gave a product free of the acid, indicating that the acid chloride starting material was of high purity.

catalysed hydrogenolysis (though it had been reported under similar conditions,[99] no cleavage of the Fmoc group was observed during this step) and the FmocGFLG purified by precipitation from THF with hexanes. It should be noted that no evidence of racaemisation was observed when crude reaction mixtures were analysed by thin layer chromatography on silica, nor when purified peptides were characterised by NMR spectroscopy. The finalised synthetic route, involving seven synthetic steps, three chromatographic steps and with an overall yield of 33%, is given below in Figure 2.14.

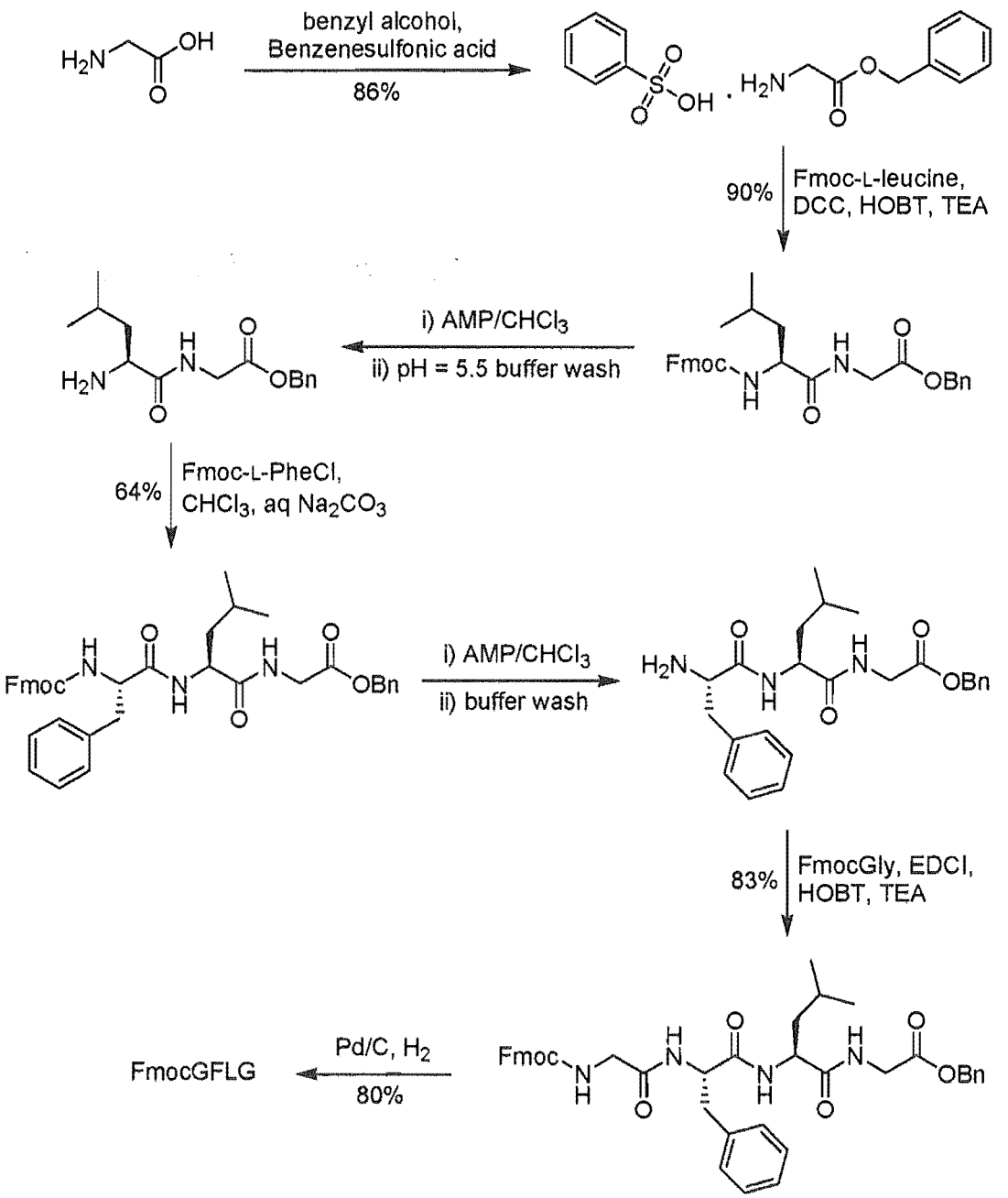


Figure 2.14: Finalised solution-phase synthetic route to FmocGFLG.

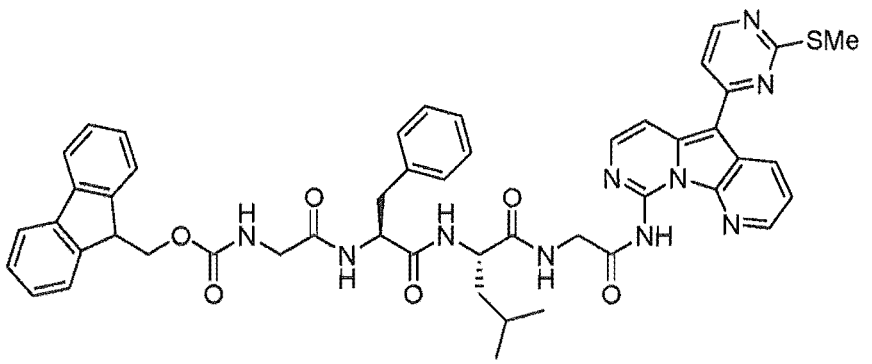
While more laborious, this synthetic route has the advantage of being robust, using a minimal amount of electrophile at each step, producing relatively clean product and enabling the efficient scale up to potentially gram-quantities.

With the biolinker in hand, attention could be turned towards the synthesis of a variolin-biolinker conjugate.

2.4.3 Acylation of **2.16** with the biolinker

2.4.3.1 DCC coupling

A DCC coupling between analogue **2.16** and FmocGFLG gave conjugate **2.18** (Figure 2.15), which precipitated out of solution during the course of the reaction. Conjugate **2.18** was isolated by filtration in 28% yield, with a small amount of contamination by DCU.



2.18

Figure 2.15: Conjugate **2.18**.

Analysis by ^{1}H NMR spectroscopy showed all the expected peptide and variolin signals. Of particular note was a signal at 12.87 ppm for the 9-NH proton, and a chemical shift of ~4.5 ppm (partially obscured by another signal) for the *C*-terminal glycine methylene group, which was significantly different from the value of 3.73 ppm seen with the free tetrapeptide. However, 2D NMR was unable to conclusively prove the structure of **2.18**, as key CIGAR correlations between the tetrapeptide and variolin core were not observed. This uncertainty was resolved by

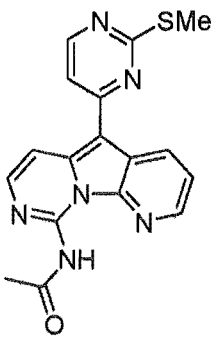
HRMS, however, which gave a strong signal for a compound of correct mass, with no observable free tetrapeptide or variolin.

The synthesis of this compound using what is ultimately an anhydride electrophile at 4 °C showed that the N(9a) position was somewhat different from other 2-aminopyrimidines, which require more agressive conditions - for example, refluxing with acetic anhydride[100-102] or using acid chlorides[103-106] - before acylation will proceed. The left hand side of Figure 2.18 gives the proposed mechanism for the acylation of N(9a), though it is not known why this reaction proceeds so much more readily than with other 2-aminopyrimidines.

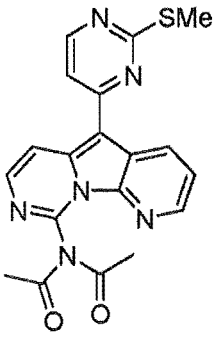
Attempts to purify conjugate **2.18** away from the DCU impurity were hindered by decomposition, to give what appeared to be analogue **2.16** by tlc analysis. Furthermore, treatment with piperidine, in an attempt to cleave the Fmoc group, led to complete decomposition to give analogue **2.16** (and, presumably, the tetrapeptide GFLG). This low stability of the *N*(9a)-acyl linkage needed to be investigated more closely. However, due to the awkward solubility and relatively high polarity of conjugate **2.18**, it was decided to investigate the stability further using a simple model system employing the acetyl moiety in place of FmocGFLG.

2.4.4 Acylation of N(9a) with AcCl

The reaction of analogue **2.16** with one equivalent of AcCl, in the presence of a slight molar excess of TEA, gave a mixture of the variolin starting material **2.16**, the monoacetyl derivative **2.19** and the diacetyl derivative **2.20** (Figure 2.16). This result was not unexpected, as the formation of derivative **2.20** under similar conditions has already been reported,[91] and the diacetylation of other 2-amino pyrimidines[101,102] is also known. Attempts to push this diacetylation reaction through to completion were unsuccessful, however, as is evidenced by the results from the reaction of **2.16** with five equivalents of AcCl, again, with a small molar excess of TEA (Table 2.1). Reactions were carried out at room temperature for 48 hours, and the variolin products purified by flash chromatography on silica.



2.19



2.20

Figure 2.16: Acetylated variolin derivatives **2.19** and **2.20**.

		Relative proportions of variolin products (%)[a]		
2.16:AcCl	Recovery (%)[a]	**2.16**	**2.19**	**2.20**
1:1	62	53	7	40
1:5	61	0	59	41

[a] Based on recovered variolin material following flash chromatography on silica.

Table 2.1: The ratio of compounds recovered from the acetlyation of **2.16** with AcCl.

Subsequent attempts to push the formation of **2.20** through to completion were unsuccessful. During the chromatography on these reactions, it was noticed that derivative **2.19** appeared to be decomposing back to **2.16** while on the silica. This was surprising, given the reported stability of other 2-acylaminopyrimidines.[103,104,106,107] For this reason the two reactions were repeated, stirring the reaction solutions at room temperature for 19 hours, and removing a 30 μL aliquot for immediate concentration *in vacuo* and analysis by ^{1}H NMR spectroscopy. In the NMR spectra of these mixtures, the three variolin products, presumably with minimal decomposition, could be quantified relative to each other by comparing the peak integral ratios of the acetyl methyl signals of derivative **2.19** (appearing at 2.676 ppm) and derivative **2.20** (both methyls were coincident and appeared at 2.41 ppm) with the *S*-methyl signal (which was coincident for all three compounds, **2.16**, **2.19** and **2.20**, and appeared at 2.685 ppm) (Figure 2.17). The results are presented in Table 2.2.

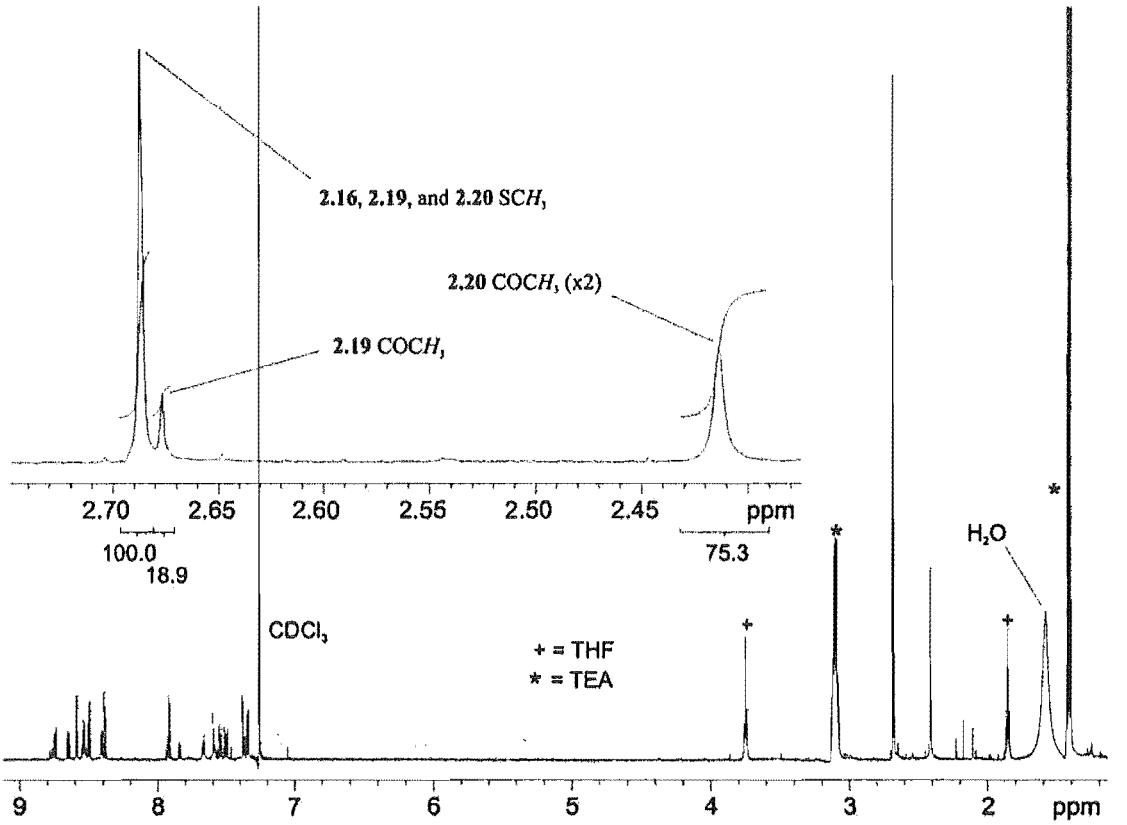


Figure 2.17: The methyl region from a ^{1}H NMR spectrum of a crude reaction mixture from the acetylation of **2.16** with 1 eq AcCl.

	Relative proportions of variolin products (%)		
2.16:AcCl	**2.16**	**2.19**	**2.20**
1:1	43	19	38
1:5	0	57	43

Table 2.2: The ratio of variolin compounds in the acetylation of **2.16**, as determined by ^{1}H NMR spectroscopy.

The NMR results confirmed what had been previously observed; namely, that regardless of the amount of AcCl present, the diacetyl derivative **2.20** is formed at about 40%. Higher stoichiometries of AcCl lead to a higher conversion of **2.16** to **2.19**, but most of this monoacetyl derivative doesn't react any further. To test this assertion, a sample of **2.19** was stirred with AcCl at room temperature for 22 h. Removal of an aliquot followed by immediate concentration *in vacuo* and analysis by ^{1}H NMR spectroscopy indicated that no acetylation had occurred (that

is, **2.20** was not present). Flash chromatography on silica gave the starting material **2.19** with 81% recovery.

As a further test, a solution of **2.20** and **2.16** stirred at room temperature for 5.5 hours gave no **2.19**, as determined by ^{1}H NMR spectroscopy. These results, taken together, prove that the product ratios given in Table 2.1 and Table 2.2 are not equilibrium concentrations.

The second conclusion is speculative and not so obvious, and that is that the acetylation of **2.19** to give **2.20** goes via a reaction catalysed by **2.16**. This assertion comes from the fact that the only obvious difference between the reactions in which **2.20** was produced and the failed attempt at acetylating **2.19** was the presence of **2.16** in the former examples. Figure 2.18 shows the proposed mechanism for the formation of **2.20**, catalysed by **2.16**. It is proposed that analogue **2.16** is acetylated by AcCl at the N-8 position to give intermediate **2.21**. This intermediate has not been detected, and it is not known how long-lived it is. Intermediate **2.21** may then undergo either an intramolecular acyl-migration to give **2.19**, or it may be subjected to an intermolecular attack by a second molecule of **2.19** to give the diacylated species **2.20**, and in the process regenerating the catalyst **2.16**. This mechanism is purely speculative, and has been formulated based only on the experimental results already given. It is not known why species **2.21** acylates **2.19** to give **2.20** while AcCl does not.

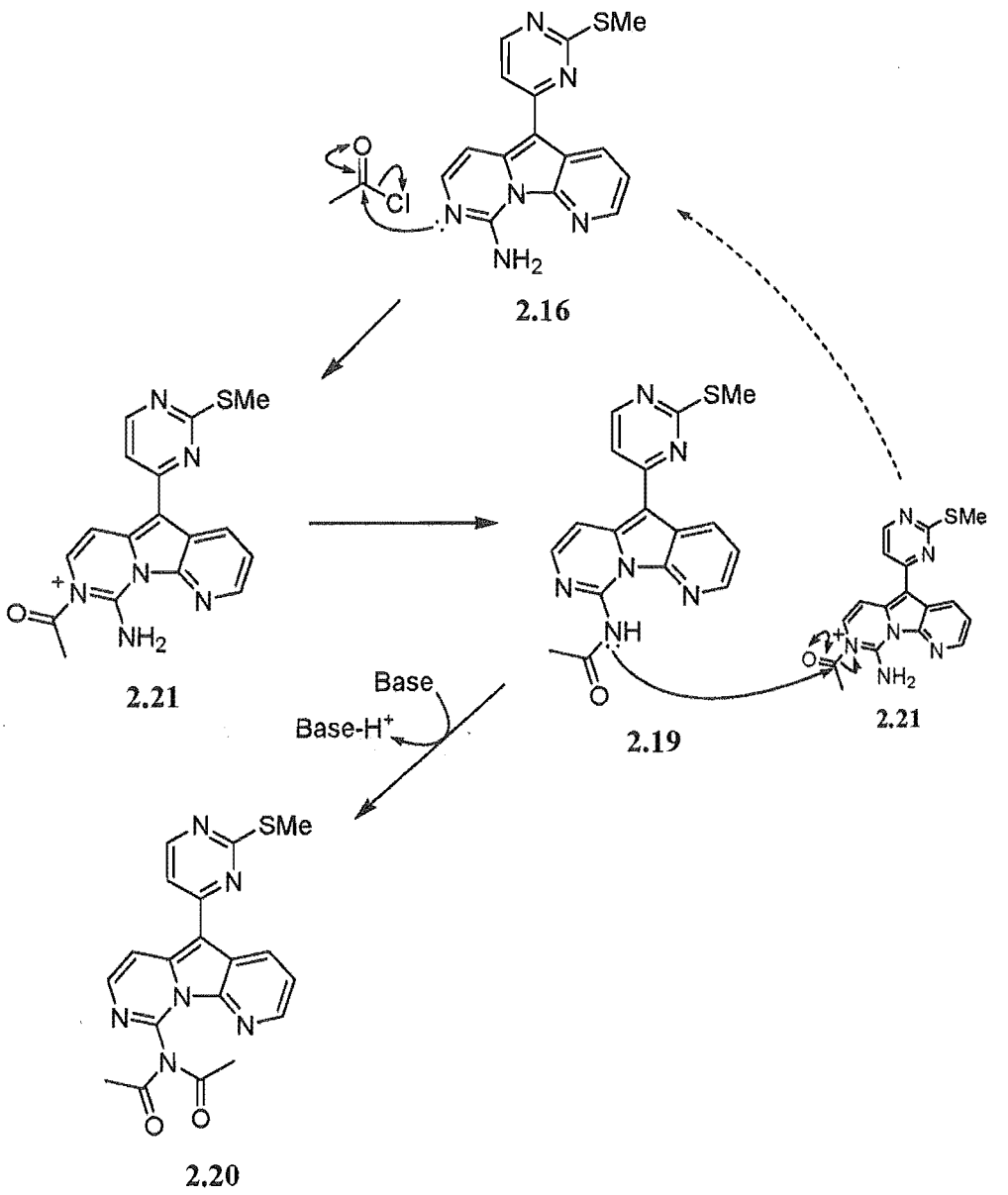


Figure 2.18: Proposed mechanism for the **2.16**-catalysed synthesis of **2.20** from **2.19**.

Conclusive proof of the catalytic involvement of **2.16** would require the use of N^{15}-labelled variolin reagents. This type of investigation, however, was considered beyond the scope of this project. Instead, a few simple experiments were done in an attempt to lend support to the proposed mechanism. The first of these involved stirring derivative **2.19**, analogue **2.16** (1 eq), AcCl (5 eq) and TEA (5.5 eq) at room temperature for 13 h. Analysis of an aliquot of the crude reaction mixture by ^{1}H NMR spectroscopy revealed that there was no remaining **2.16** starting material, with a ratio of **2.19** to **2.20** of 75:25. Given that **2.19** will not be acylated in the absence of **2.16**, and **2.16** alone will give ~40% **2.20** in the reaction with excess AcCl, it can be inferred that some intermolecular catalysis has occurred, that is, if the **2.19** starting material was

not acylated and did not participate in the formation of **2.20**, then 40% acylation of **2.16** would be expected to give a final ratio of **2.19** and **2.20** of 80:20. The observed ratio was 25% higher than that expected for the hypothetical reaction in which no **2.19** starting material reacts to form **2.20**, which is considered to be above the margin of error for the experiment. It is therefore proposed that the presence of **2.16** allowed the **2.19** starting material to be acylated to give **2.20**.

A second experiment involved repeating the above experiment, with the difference that the bis-thiomethyl core structure **2.6** was substituted for analogue **2.16**. No formation of **2.20** was observed in this experiment, indicating that core structure **2.6** does not form an acylating species in the same way as analogue **2.16** forms **2.21**.

A final investigation into this reactivity involved a time course experiment. This involved the reaction of analogue **2.16** with AcCl (5 eq) and TEA (5.5 eq), with aliquots periodically being removed, diluted with THF, injected onto the HPLC and analysed at 256 nm. For each run the peak areas of **2.16**, **2.19** and **2.20** were measured and directly compared with each other (for the purpose of this quick experiment, the extinction coefficients of the three compounds at the specified wavelength were considered to be comparable). The changing proportions of the three variolin compounds during the first 20 minutes is shown below in Figure 2.19.

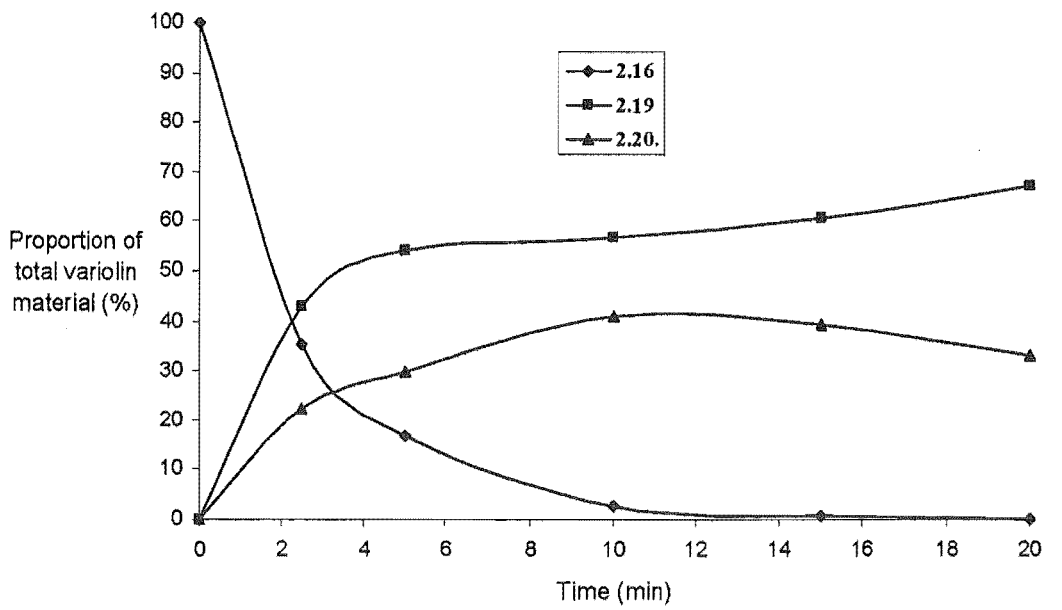


Figure 2.19: An HPLC-based time course investigation for the acetylation of **2.16** with AcCl.

It can be clearly seen that the reaction is complete in about the first 10 minutes. At 5 minutes 80-85% of **2.16** has reacted, with ~30% **2.20** and ~55% **2.19** having been formed. In the next five minutes the remaining **2.16** is all but completely consumed with an increase in the relative concentration of **2.20** of 10-15%, while the relative concentration of **2.19** is seen to increase only slightly. This is presumed to be due to the fact that **2.19** is being consumed (to form **2.20**) almost as quickly as it is being formed (from **2.16**). In the final 10 minutes, following complete consumption of **2.16**, no more **2.20** (whose rate of formation at any time is seen to be a function of the concentration of **2.16**) is formed; in fact, a small amount of hydrolysis of **2.20**, to give **2.19**, is observed, presumably through traces of water that were introduced by the repeated removal of aliquots with the same syringe (see Section 2.4.5 for a discussion of the decomposition of **2.20** to **2.19**). These results are entirely consistent with the proposed mechanism shown in Figure 2.18.

2.4.5 Stability of derivatives **2.19** and **2.20**

Varying levels of instability of both monoacetate **2.19** and diacetate **2.20** had been observed, with both breaking down to give **2.16** (and **2.20** first breaking down to give **2.19**, which accumulates under basic conditions), and so it was decided to look more closely at this. The first investigation was simple - successively larger amounts of TFA (chosen because of its use in reversed phase purification) or piperidine (chosen because of its use in Fmoc-deprotection) were added to solutions of **2.19** or **2.20** in THF and the solutions periodically analysed by tlc. Efforts were not made to exclude water. Results are given below in Table 2.3 and Table 2.4.

[TFA] (v/v)	0.1%		1.0%		10%	
Time (min)	30	120	30	120	30	120
2.19 decomposition	partial	partial	complete	-	-	-
2.20 decomposition	nil	nil	minimal	partial	complete	-

Table 2.3: Degradation of **2.19** and **2.20** to **2.16** in TFA/THF solutions.

[Piperidine] (v/v)	0.1%		1.0%		10%	
Time (min)	30	120	30	120	30	120
2.19 decomposition	minimal	minimal	partial	partial	complete	-
2.20 decomposition	complete	-	-	-	-	-

Table 2.4: Degradation of **2.20** to **2.19**, and **2.19** to **2.16** in piperidine/THF solutions.

Both **2.19** and **2.20** readily break down to **2.16** (as indicated by tlc) within 30 minutes in 10% (v/v) solutions of TFA or piperidine. This is in contrast to most 2-amidopyrimidines, which typically require much more vigorous conditions for the removal of acyl groups.[107] **2.20** breaks down to **2.19** very quickly in the presence of piperidine, whereas it shows significantly higher stability towards TFA, breaking down to **2.16** only at higher concentrations. **2.19**, while not as stable as **2.20** towards TFA, is significantly more stable towards piperidine, more stable, in fact, than it is towards TFA. The order of decreasing stability, then, is **2.20** (TFA) > **2.19** (piperidine) > **2.19** (TFA) > **2.20** (piperidine). This is an interesting result, and so will be discussed here in more detail.

One would expect **2.20** to be less stable than **2.19** simply due to the higher reactivity of imides over amides. However, this is not the case for the decomposition in acidic conditions. There are most probably several important factors explaining these differences in stability between the two in both acid and base, which are outlined below.

The probable mechanism of the aminolysis of **2.20** by piperidine is given in Figure 2.20. **2.20** is shown with the acetyl groups out of the plane of the ring (a steric requirement). Decomposition is proposed to proceed via a direct nucleophilic attack on an acetyl carbonyl, followed by expulsion of the resonance-stabilised anionic variolin leaving group. (What is not shown in Figure 2.20 is that this resonance stabilisation may also involve the delocalisation of the negative charge into the variolin core structure, in an analogous fashion to that shown in Figure 2.22.) In support of this mechanism of decomposition is the observation that only minimal degradation of **2.20** was seen to occur during chromatography on silica with eluants containing 0.1% (v/v) TEA. TEA, while being a stronger base than piperidine, is not nucleophilic.

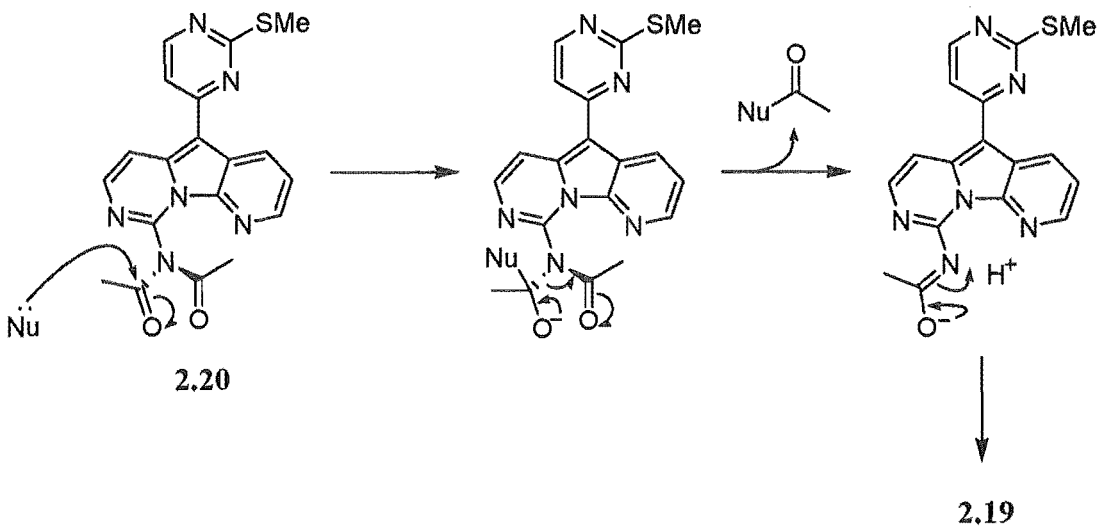


Figure 2.20: Nucleophilic attack on **2.20** to give **2.19** under basic conditions.

Aminolysis of **2.19** is also proposed to proceed via a direct nucleophilic attack at the acetyl carbonyl, where a strongly hydrogen-bonded 9-NH proton (as is evidenced by the ^{1}H NMR spectrum of this compound) ensures that the acetyl moiety lies in the plane of the ring. The tetrahedral intermediate will then break down to give an anionic variolin leaving group, which will in turn be protonated to form **2.16** (Figure 2.21).

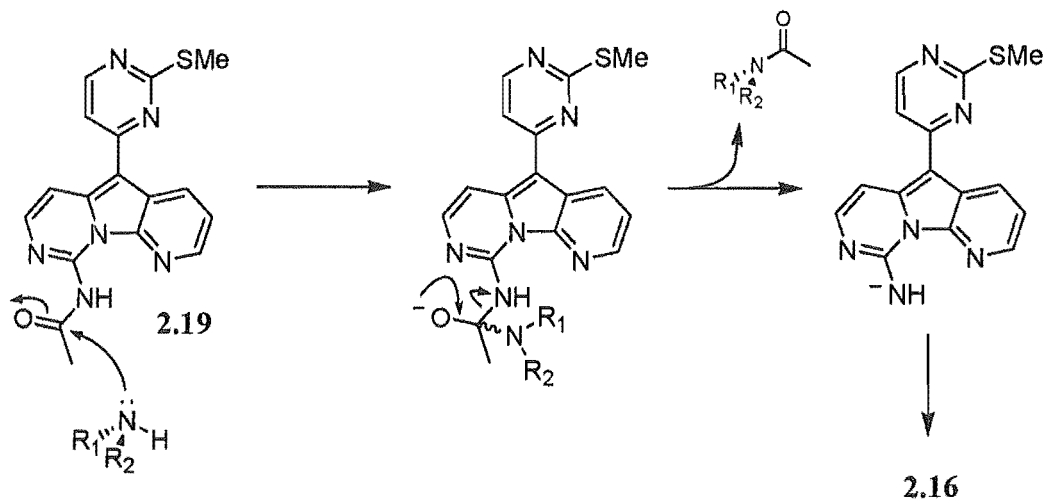


Figure 2.21: Aminolysis of **2.19** to give **2.16**.

As has already been pointed out, this sort of lability is not normally observed in 2-aminoacyl pyrimidines. It is proposed, therefore, that the reactivity of **2.19** can be attributed to the stability of the anionic variolin leaving group, a species in which the negative charge may be delocalised

across 2 different nitrogens (Figure 2.22). The observed aminolytic lability of **2.19** also indicates that the 'degree' of aromaticity of the bottom pyrimidine ring is less than that of more conventional 2-aminopyrimidines (including the 'top' pyrimidine ring of DVB – see Section 2.5.3), and so resonance stabilisation (with the associated loss of aromaticity) is less energetically costly.

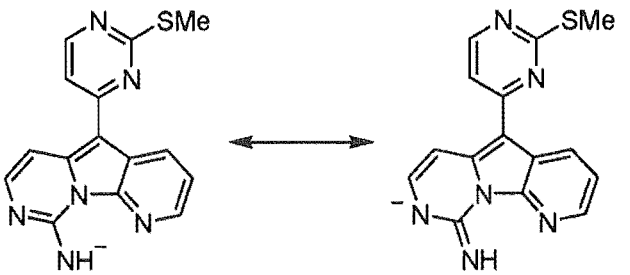


Figure 2.22: Resonance stabilisation of the anionic variolin leaving group.

A proposed mechanism for nucleophilic attack on **2.19** under acidic conditions first involves protonation of the variolin core. There are a total of five nitrogens in **2.19** that could be protonated, and under acidic conditions the protonated forms of all will be in equilibrium with one another. Figure 2.23 shows that protonation of N-8 will activate the acetyl group for nucleophilic attack due to hydrogen bonding between the acidic proton and the carbonyl oxygen. Following nucleophilic attack to give the tetrahedral intermediate, N-8 may then participate again in the acceptance of the same proton from the hydroxyl group in a concerted decomposition reaction that leads to expulsion of **2.16**. Alternatively, decomposition may proceed like that shown in Figure 2.21 with the expulsion of a resonance-stabilised anionic variolin leaving group (Figure 2.22). Again, the surprising reactivity of this compound presumably reflects the lower 'degree' of aromaticity, and therefore higher energy, of the bottom ring system (that is, when compared with more conventional 2-aminopyrimidines).

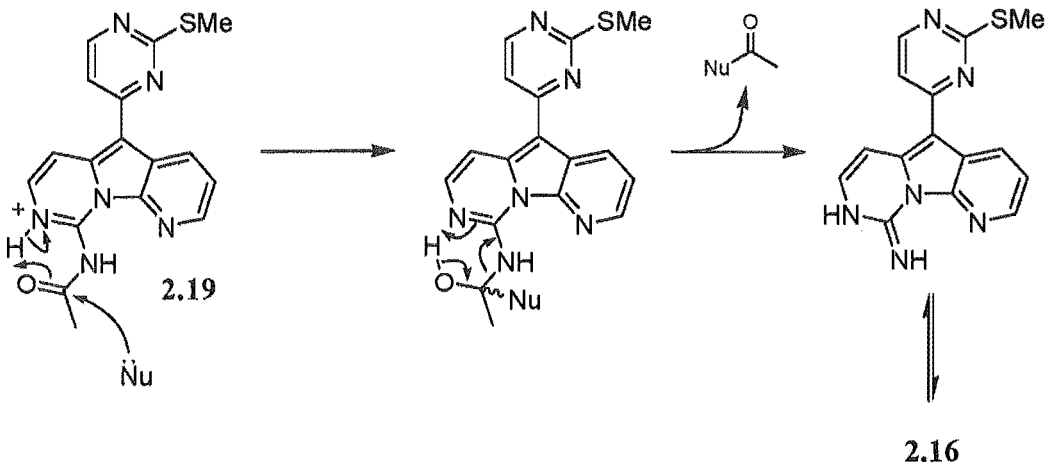


Figure 2.23: Acid-catalysed hydrolysis of **2.19**.

In the experiment described above (TFA-catalysed decomposition) the nucleophile was water, traces of which would have entered the THF reaction solution during the course of the reaction.

The observation that **2.20** is hydrolysed more slowly than **2.19** under acidic conditions can be explained by the assertion that a protonated form of **2.20** is not able to form the type of hydrogen bond (between the proton on N-8 and the carbonyl oxygen) shown in the structure on the left hand side of Figure 2.23 due to steric clash between the methyl of the other acetyl group and N-1. Put another way, the steric clash with N-1 forces the acetyl moieties of **2.20** out of the plane of the ring, which disallows the carbonyl oxygens to act as hydrogen bond acceptors for the protonated N(8)-H hydrogen bond donor.

As derivative **2.19** was a better model for the type of polymer therapeutic conjugates that were the target of this work, its stability in aqueous solution was investigated more closely. Thus, solutions of **2.19** (22 μM) in 3:1 DMSO/aqueous buffer solutions were left at 30 °C, and aliquots periodically removed and analysed by HPLC at 256 nm. The peak areas for **2.16** and **2.19** were measured and directly compared (no adjustment was made for the likely difference in extinction coefficients at the specified wavelength). The results for three separate reactions at pH = 5.0, 7.4 and 9.3 are given below in Figure 2.24.

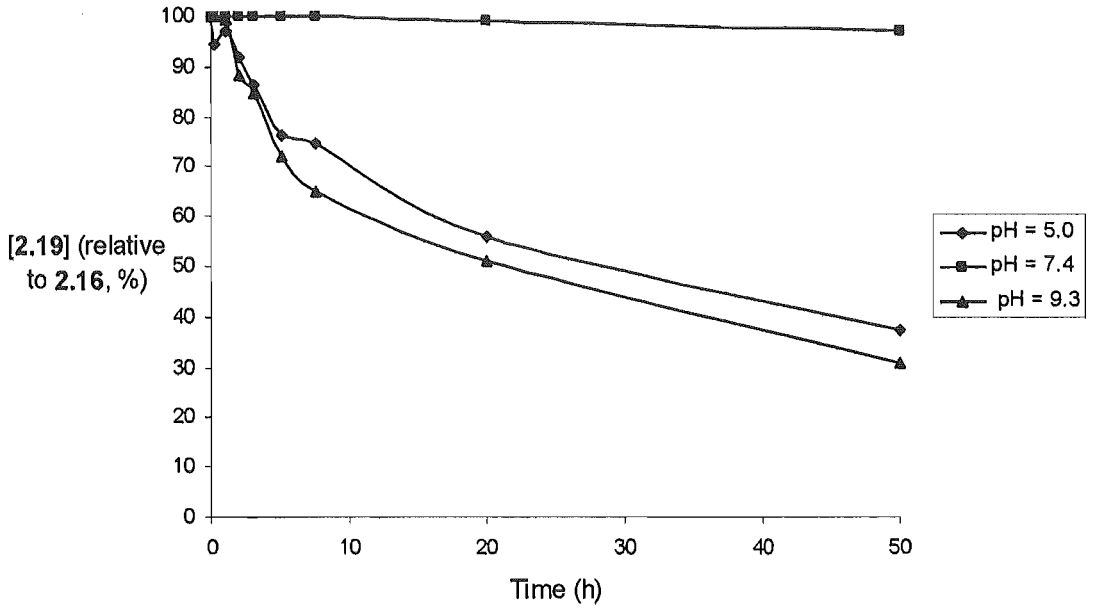


Figure 2.24: The pH-dependent decomposition of **2.19** to **2.16** in aqueous solutions.

At physiological pH, 3% degradation had occurred after 50 h, whereas 63% and 69% degradation had occurred at pH = 5.0 and 9.3, respectively. This result was double checked by repeating the experiment, using THF in the place of DMSO, and analysing the solutions only once, after 43 h. The results are given below in Table 2.5.

pH	% Decomposition*
5.0	30
7.4	2
9.3	90

* Determined by comparing the peak areas of **2.19** with **2.16**.

Table 2.5: Decomposition of **2.19** in aqueous THF solutions of varying pHs.

These results were similar to the earlier findings, albeit with a larger observed difference in decomposition between pHs 5.0 and 9.3.

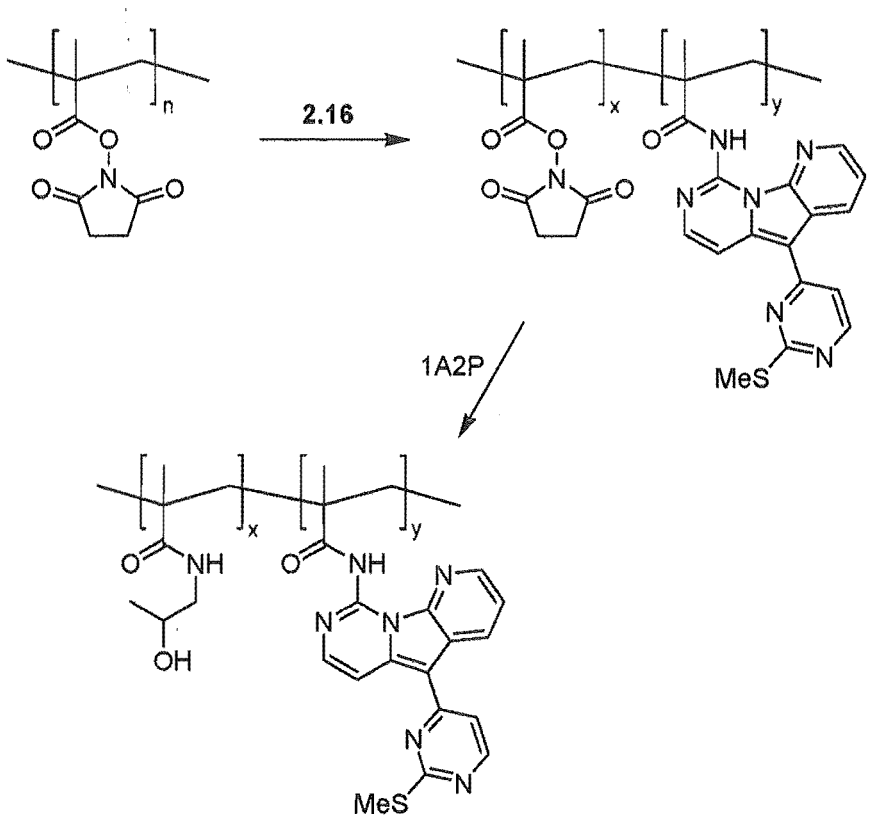
The observed acid-catalysed bond cleavage could be useful in the context of polymer therapeutics - a polymer carrying analogue **2.16**, directly bound via N(9a), will be stable in the

blood plasma (pH ~7.4) but unstable in the relatively acidic lysosomal environment (pH ~4.8), thus leading to intracellular liberation of free **2.16**. Furthermore, the interstitial medium of tumours is often acidic (due to secretion of lactic acid by cancer cells in what is often an hypoxic environment),[5] so that the drug may even be liberated from the polymer backbone in the interstitial space. Diffusion of small molecules in the interstitium will be much faster than that of macromolecules,[5] thus allowing the drug to reach its target more rapidly (see Section 1.1.2.1).

Efforts were therefore turned towards the synthesis of a pHPMA-based polymer therapeutic carrying analogue **2.16** directly bound to the polymer backbone.

2.4.6 Reaction of **2.16** with pMAOS

Several attempts were made to synthesise polymer therapeutic **2.22** via the protocols shown in Figure 2.25.



2.22

Figure 2.25: Attempted synthesis of polymer therapeutic **2.22**.

During the workup of the reaction solutions following the first step, however, it became obvious that analogue **2.16** was not bound to the polymer backbone. Analyses using ^{1}H NMR spectroscopy confirmed this. The most likely explanation was that **2.16** was being lost from the polymer intermediate due to attack by 1A2P at the methacrylate carbonyl of variolin-bearing polymer units. However, purification of the polymer intermediate following the first step showed that most of the variolin starting material was not polymer bound, even before the addition of 1A2P. It was therefore considered desirable to develop a simple small-molecule model system to investigate this reaction more closely.

2.4.7 A model system for **2.22** syntheses

The simple pMAOS analogue *N*-hydroxysuccinimidyl pivalate, **2.23**, (Figure 2.26), a known compound,[108] was synthesised from pivaloyl chloride and *N*-hydroxysuccinimide (NHS). Reaction of analogue **2.16** with ten equivalents of **2.23** gave mixtures of the desired product **2.24** (Figure 2.26) and the two starting materials, favouring the starting materials ~2:1. A temperature of 100 °C was needed for the reaction to proceed in reasonable time. The observation that the reaction couldn't be pushed to completion (temperatures of 130 °C led to the decomposition of the variolin starting material), taken together with the fact that the two starting materials were always seen in the ^{1}H NMR spectrum of the crude reaction mixtures, suggested that an equilibrium was being established (Figure 2.26).

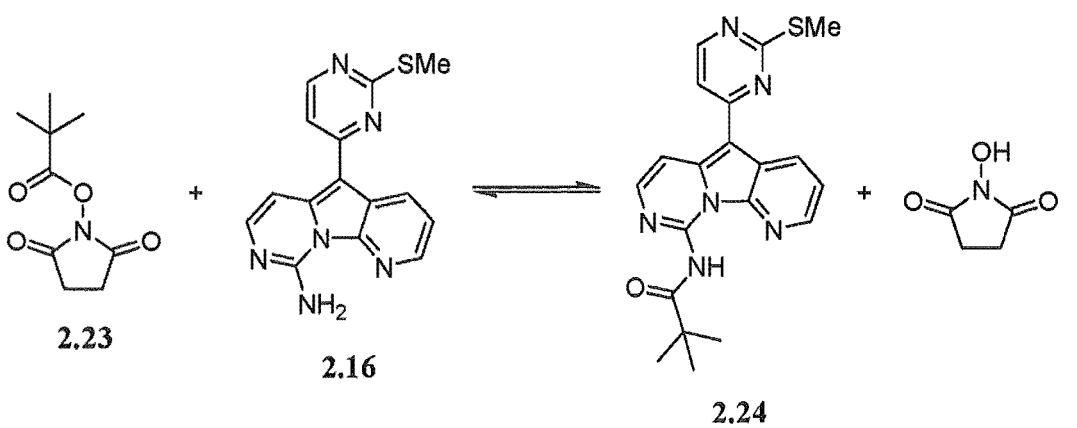


Figure 2.26: The equilibrium between **2.16**, **2.23**, **2.24**, and NHS.

The possibility that an equilibrium was being established was investigated by heating **2.24** with one equivalent of NHS under the same conditions as for its formation (water was carefully excluded). ^{1}H NMR spectroscopic analysis clearly showed the presence of both the variolin starting material and the products **2.16** and **2.23**, favouring the products ~3:1, thus confirming the proposed equilibrium. All three compounds were isolated by flash-chromatography on silica.

The discovery that analogue **2.16** had comparable leaving group ability to NHS (possible acid catalysis by NHS not withstanding) indicated that the proposed protocols for the synthesis of a polymer therapeutic carrying analogue **2.16** directly bound to the polymer would not be suitable. Specifically, it was realized that the second synthetic step involving two equivalents of 1A2P would certainly lead to aminolysis of the variolin-polymer bond. This rationale would also hold true for a more "conventional" polymer therapeutic construct carrying a variolin-tetrapeptide conjugate. Attempts to synthesise a polymer therapeutic carrying a deoxyvariolin analogue, bound via the N(9a) position, were therefore abandoned, and attention was instead focussed on the N(2'a) position.

2.5 Acylation of N(2'a)

2.5.1 Synthesis of *N*(9a)-protected DVB

Bis-PMB-protected DVB, **2.26**, was synthesised according to protocols developed by Anderson and Morris.[85,87] Thus, oxidation[109] of core structure **2.6** to give the bis-sulfoxide derivative, **2.25**, followed by reaction with *p*-methoxybenzylamine (PMB), gave **2.26** in 72% yield over two steps (Figure 2.27).

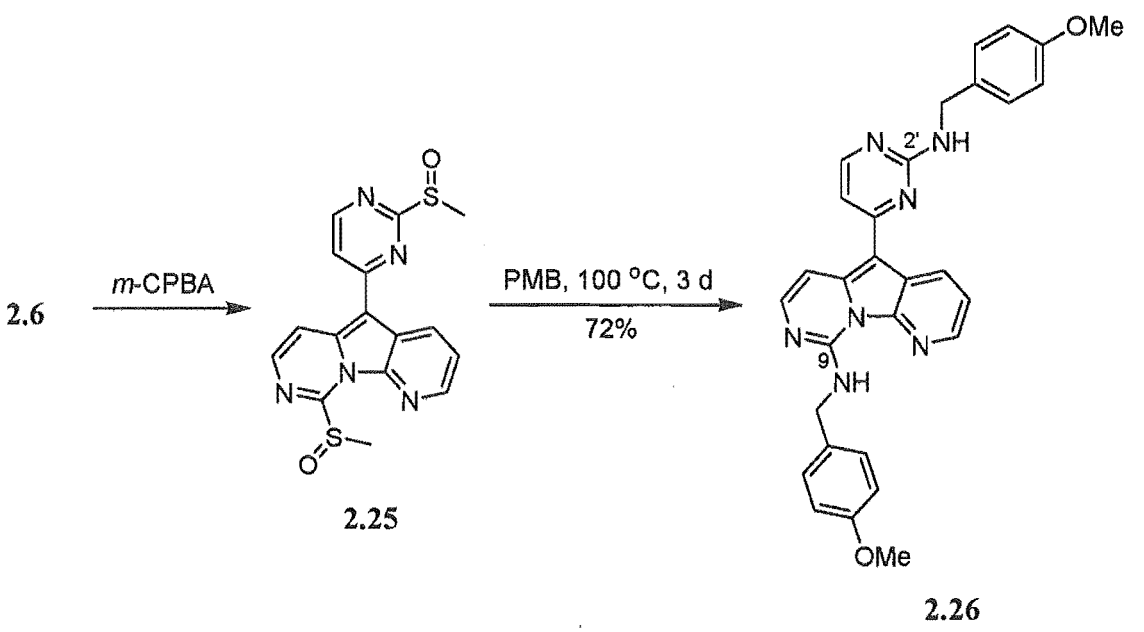


Figure 2.27: Synthesis of **2.26** from **2.6**.

The selective deprotection of the 2'-PMB group from a bis-PMB-protected variolin analogue had already been demonstrated by Anderson and Morris in the methoxyvariolin series,[87] and so similar protocols were applied in this work in the mono-deprotection of analogue **2.26**. Thus, **2.26** was heated in neat TFA at 45 °C for 24 h to give analogue **2.27** in 83% yield (Figure 2.28).

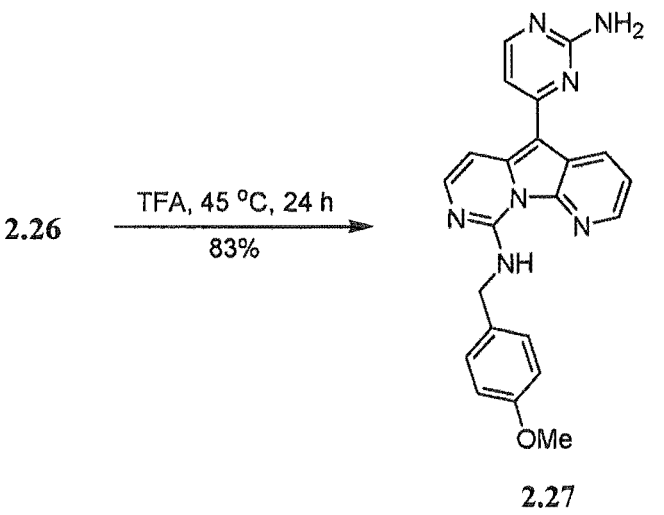


Figure 2.28: Synthesis of **2.27** from **2.26**.

The bioactivity of **2.27** against the P388 cell line was found to possess an IC_{50} of 5.4 μM, around 10 times less bioactive than DVB. Incorporation of this analogue into a polymer therapeutic was

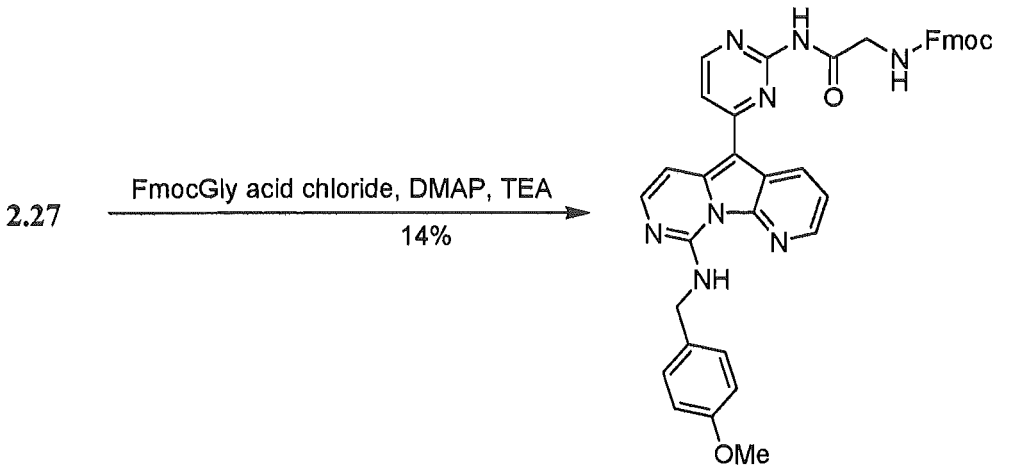
therefore pursued, but considered a less important target than a polymer therapeutic carrying DVB bound via the N(2'a) position.

2.5.2 Acylation of **2.27**

As has been already mentioned (Section 2.4.3.1) the acylation of 2-aminopyrimidines usually requires relatively strong conditions, such as boiling in acetic anhydride or reacting with acid chlorides. It was not surprising, therefore, to discover that the DCC/HOBT, EDCI/HOBT and HBTU coupling systems did not lead to coupling between **2.27** and FmocGFLG.

Attention was therefore turned towards acid chloride chemistry. Attempted synthesis of FmocGFLG acid chloride from the reaction of FmocGFLG with oxalyl chloride, however, led to consumption of the tetrapeptide in an unidentified side reaction before the variolin nucleophile was added (see below, Section 2.6.2.1). A proposed synthetic route, then, involved the synthesis of a **2.27**-glycine conjugate, **2.29** (Figure 2.30) followed by acylation of this intermediate with FmocGFL. This variolin tetrapeptide conjugate, following Fmoc deprotection, could then be reacted with pMAOS to give a polymer therapeutic carrying analogue **2.27**. Alternatively the PMB group could first be deprotected to give a DVB-tetrapeptide conjugate, which would then be reacted with pMAOS to give a DVB-carrying polymer-drug construct of type **1.1** (polymer cross-linking via the *N*(9a) of DVB would be avoided due to the lability of this bond, as has been outlined above in Section 2.4.7).

Analogue **2.27** was reacted with one equivalent of Fmoc-glycine acid chloride in a reaction which gave a mixture of variolin compounds, including the starting material (as determined by tlc). Increasing the stoichiometry of the acid chloride to four equivalents appeared to do little in the way of pushing the reaction to completion, with variolin starting material remaining upon completion of the reaction. The desired monoacylated product, **2.28**, was isolated from one of the reaction mixtures in 14% yield (Figure 2.29).



2.28

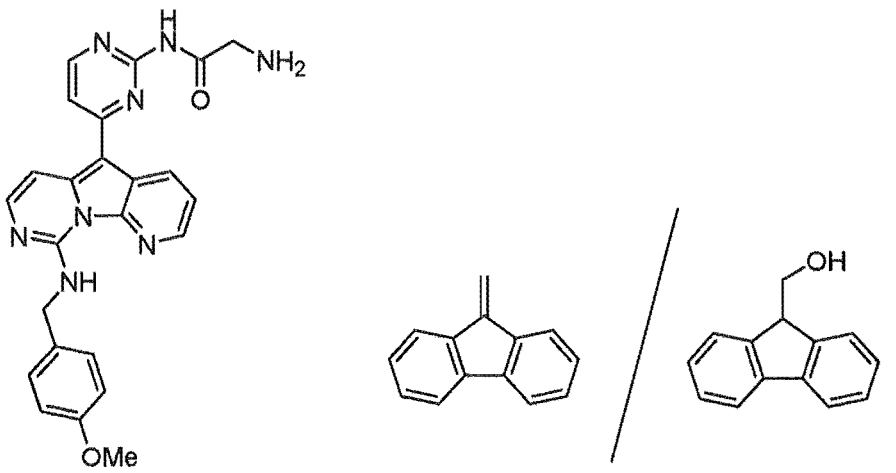
Figure 2.29: Synthesis of **2.28** from **2.27**.

It is not known why this reaction did not proceed more cleanly, nor why starting material remained in solution despite large excesses of the electrophile. Decomposition of **2.27** on the tlc plates would not have been occurring to any significant extent (see Section 2.5.3). The side products were not isolated and characterised, with this work being abandoned before further efforts were made towards its optimisation (see Section 2.5.3).

2.5.3 Stability of *N*(2'a)-acyl linkage

The stability of the *N*(2'a)-acyl linkage was investigated. A solution of monoacylated variolin **2.28** in 5% TFA/DMSO showed no appreciable decomposition after 5 hours at room temperature when analysed by HPLC (**2.28** elutes at 15.1 minutes using the standard gradient elution protocols). The *N*(2'a)-acyl linkage is therefore much more stable towards acid than the *N*(9a)-acyl linkage.

The experiment was repeated with TEA in the place of TFA – decomposition was observed by HPLC, but was presumed to be due to Fmoc deprotection. This decomposition gave a variolin compound eluting at 9.8 minutes (using standard gradient elution protocols), presumed to be **2.29** (**2.27** elutes at 11.4 minutes), and a compound eluting at 20.9 minutes, presumed to be one of two Fmoc-deprotection fulvene species' (Figure 2.30). Fmoc deprotection by TEA has been previously reported.[110]



2.29

Figure 2.30: Proposed **2.28**-deprotection products detected by HPLC.

In support of these proposed structures, **2.28** was stirred in 20% piperidine/THF for 15 minutes then analysed by HPLC to show the same two compounds eluting at 9.8 minutes and 21 minutes, respectively. Despite this promising result, **2.29** was not isolated nor further characterised, due to results that are described below.

In a parallel investigation **2.28** was stirred in neat triflic acid for 2 h, the variolin material purified using a C_{18} cartridge, and the crude mixture analysed by HPLC. Perhaps not surprisingly, cleavage of the *N*(2'a)-acyl linkage had accompanied PMB deprotection to give DVB (eluting at 7.2 minutes).

Alternative PMB-deprotection strategies had been investigated by Anderson and Morris, and had proven problematic. Rather than revisit this chemistry, it was decided to pursue the acylation of DVB directly. The logic behind this was that, while acid chloride electrophiles had been shown to acylate both N(9a) and N(2'a), the *N*(9a)-acyl group(s) could then be selectively cleaved in the presence of the *N*(2'a)-acyl groups.

2.6 Acylation of DVB

2.6.1 DVB Synthesis

Efforts were initially focussed towards scaling up the synthesis of DVB developed by Anderson and Morris.[87] PMB-deprotection of **2.26** with neat triflic acid gave DVB in reasonably high purity as a yellow/brown solid. Several strategies were tried in the purification of this material, but most were hindered by the relatively high polarity and low solubility of DVB. Eventually, protocols were established whereby a small amount of DIOL was added to a ~2 mg/mL solution of the crude DVB in THF. This slurry was then diluted slowly and with vigorous swirling with ~1.5 eq volumes of hexanes, leading to the coating of the DIOL with DVB. This yellow slurry was then loaded onto a DIOL column and the DVB eluted with 2% MeOH/CH_2Cl_2. The DVB eluted slowly, over 20-30 fractions (~10 column volumes), but was obtained in high purity nonetheless with a yield of 88%. The IC_{50} of this material was found to be 0.47 μM against the P388 cell line.

2.6.2 Acylation with acid chlorides

2.6.2.1 Tetrapeptide acid chloride

Acylation of DVB by FmocGFLG acid chloride was attempted. Oxalyl chloride was added to a solution of FmocGFLG in THF - the evolution of gas was observed, indicating that the acid chloride had been formed. The presumed acid chloride was worked up and then reacted with DVB. However, analysis of the crude reaction mixture by HPLC (using the standard gradient elution protocol) showed only two compounds – DVB (eluting at 7.3 minutes) and an unknown compound eluting at 18.5 minutes (the FmocGFLG starting material elutes at 15.3 minutes) and possessing an Fmoc chromophore. This material was partially purified on silica, and analysis by ^{1}H NMR spectroscopy showed it to be peptidic (though clearly not the FmocGFLG starting material); specifically, Fmoc aromatic and leucine methyl signals were unambiguously identified.

While the FmocGFLG acid chloride decomposition side product was not characterised, it was clear that the electrophile was being consumed by some side reaction. For this reason it was

decided to investigate an alternative synthetic pathway. Fmoc-glycine acid chloride was already in hand, and so it was decided to acylate the relatively unreactive N(2'a) position with this electrophile, deprotect the Fmoc group, then acylate again with FmocGFL using conventional solution-phase peptide coupling protocols (an EDCI coupling, for example). The N(9a) position would also acylate at each of the two steps, but the resulting bond could be easily cleaved with acid or base, while preserving the *N*(2'a)-peptide linkage (Figure 2.31).

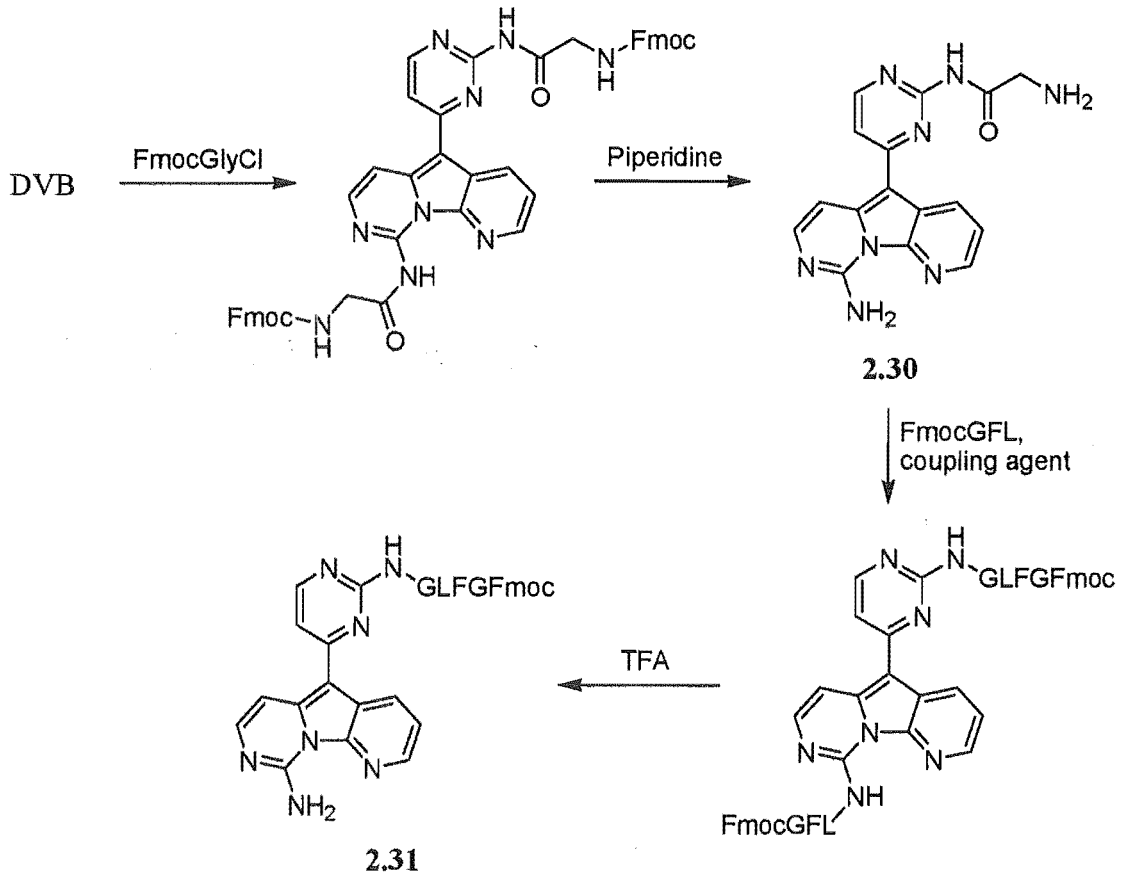


Figure 2.31: Proposed synthetic pathway to **2.31**.

It was anticipated that purification of analogue **2.30** would be difficult, given the high polarity of both **2.30** itself and the side products from the deprotection reaction (specifically, glycine). It was envisioned that **2.31** would be purified by reversed-phase semipreparative HPLC. Deprotection of the Fmoc group of **2.31** in a piperidine/THF solution followed by addition of hexanes to give the free amine conjugate as a precipitate would be expected to proceed without complication.

2.6.2.2 *FmocGFL tripeptide synthesis*

Conventional solution-phase protocols were again employed in the synthesis of the tripeptide FmocGFL. Initial attempts to synthesise the tripeptide in a *C-N* direction were again met with problems of decomposition during the dipeptide deprotection step. While the deprotection side-product wasn't characterised, it could be clearly seen as a separate set of signals in the ^{1}H NMR spectrum of the crude reaction material, and was again presumed to be the diketopiperazine derivative (Figure 2.32). Approximately 30% of the starting material had cyclised following deprotection.

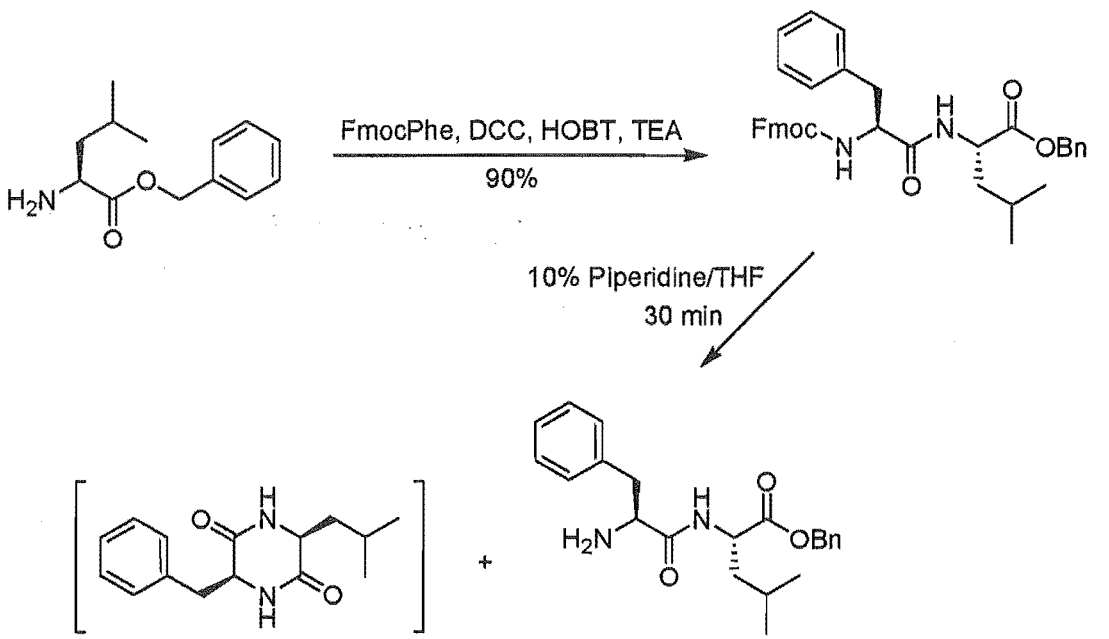


Figure 2.32: Diketopiperazine formation in *C-N* dipeptide solution-phase synthesis.

It was therefore decided to synthesise the tripeptide in an *N-C* direction. The synthetic route developed is shown below in Figure 2.33. The dipeptide intermediate FmocGF could have been synthesised by a more direct route involving the reaction between phenylalanine and Fmoc-glycine acid chloride, though concerns regarding the ease of purification of this compound led to the slightly more laborious route being taken. The synthesis proceeded without complication (though, the synthesis of the L-phenylalanine benzyl ester proceeded in only 26% yield), to give the desired tripeptide FmocGFL in 5 steps and 46% overall yield.

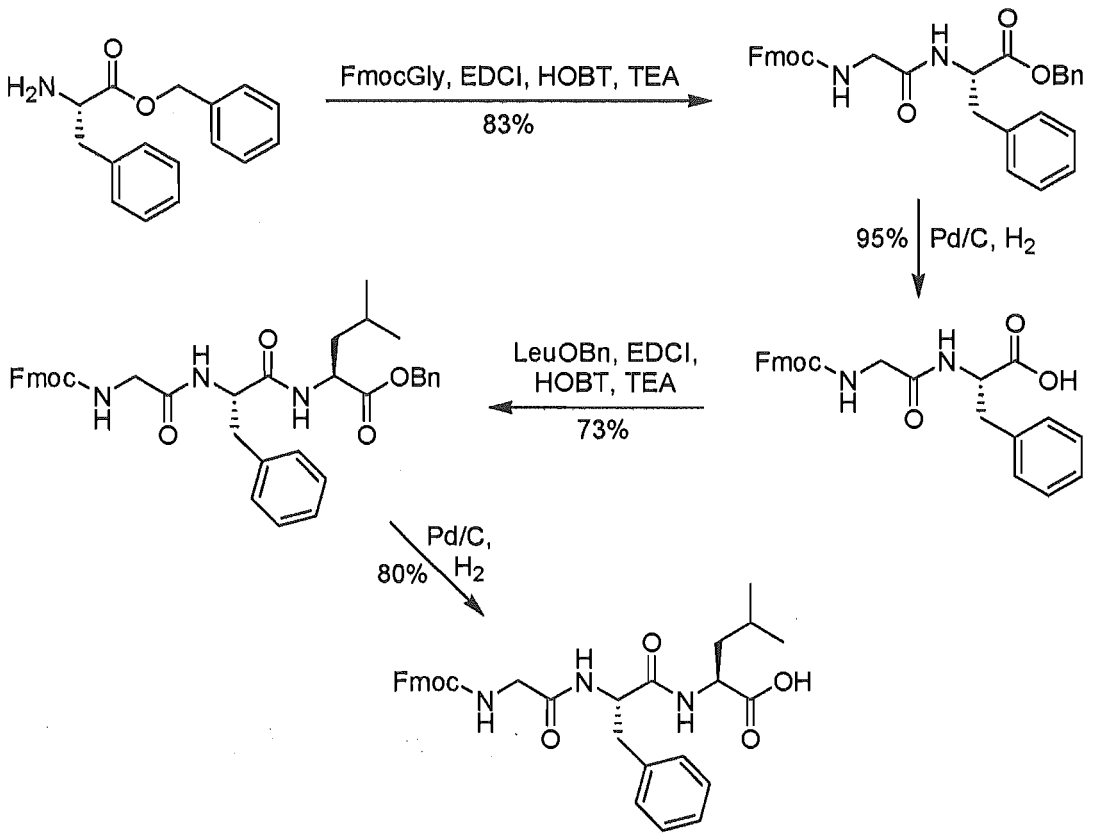
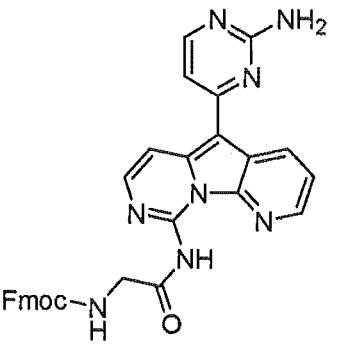


Figure 2.33: *N-C* tripeptide solution-phase synthesis.

2.6.2.3 Acylation of DVB by Fmoc-glycine acid chloride

During the synthesis of the tripeptide, an investigation involving the acylation of DVB by Fmoc-glycine acid chloride was also being carried out. The reaction between DVB and one equivalent of Fmoc-glycine acid chloride gave predominantly one monoacylated derivative, as determined by LCMS. As a quick test of the identity of this acylated derivative, a small amount of TFA was added to the crude reaction mixture, which was then left at room temperature for ten minutes before being analysed again by HPLC. The HPLC trace showed that that the conjugate had completely broken down to DVB and Fmoc-glycine, indicating that the acylated compound was most probably the *N*(9a)-acylated conjugate **2.32** (Figure 2.34). This assertion that the N(9a) position had been acylated in preference to the N(2'a) position was consistent with the observed differences in the ease of acylation of these sites (see Sections 2.4.3.1 and 2.5.2). The result also confirmed an earlier result obtained with 2'-thiomethyl analogue **2.16** indicating that the N(9a) position will only be acylated once by the Fmoc-glycine acid chloride electrophile. It is not known why diacylations do not occur with this electrophile, but it may be due to the

increased steric hindrance around the carbonyl electrophilic centre (compared with acetyl chloride).



2.32

Figure 2.34: **2.32**, the kinetic product from the reaction between DVB and Fmoc-glycine acid chloride.

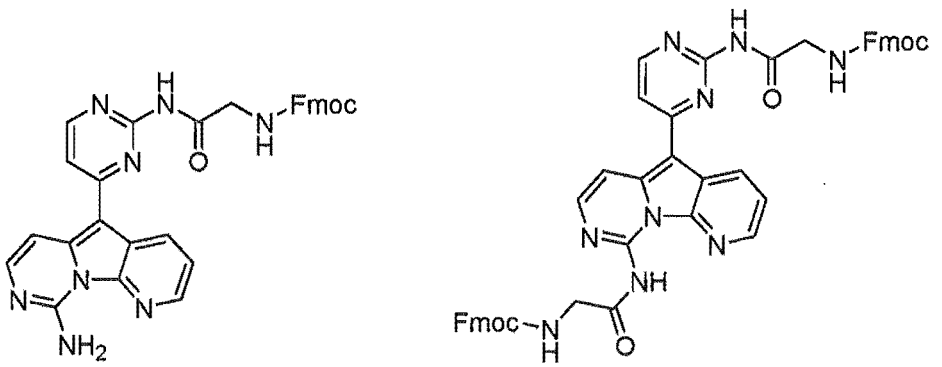
As expected, the reaction between DVB and Fmoc-glycine in an EDCI-mediated coupling also gave only **2.32**. It was hoped that a solution of this analogue heated to sufficiently high temperatures may rearrange to give **2.33** (see Figure 2.35). It was envisioned that this reaction would go via an intermolecular attack by N(2a) of one variolin molecule on the acyl carbonyl of **2.32**, to give a molecule of DVB and **2.33**, the latter of which would not be expected to react any further. At temperatures >100 °C, however, decomposition of **2.32** occurred without the formation of any **2.33**. It is not known what exactly was taking place, but the most likely possibility is that Fmoc deprotection by the basic variolin core was occurring (see Section 3.2 for a comparable result) to give glycine residues that would react as nucleophiles in preference to the variolin 2'-NH_2 group. This idea was not explored in any more detail.

DVB acylation reactions involving higher stoichiometries of the acid chloride electrophile were carried out. Analysis by HPLC of the crude reaction mixtures consistently showed that a mixture of products had been formed, and DVB starting material was always observed (despite the use of anhydrous techniques that had been shown to be effective in other systems employing acid chlorides). LCMS was able to show that two separate monoacetylated and one diacetylated conjugate had been formed, indicating that the N(2'a) position was being acylated, though not to completion.

It is not known why these acylation reactions were so awkward, neither proceeding to completion nor giving a single product, regardless of the stoichiometry of the electrophile. One

possibility is that protonation of the variolin nucleophile by liberated HCl may diminish its nucleophilicity. An excess of TEA had been included in the reaction mixture to address this possibility, but the relative basicities of TEA and DVB are not known. In part response to this issue, pyridine was employed as the solvent in the place of THF in the hopes that it would act as a proton "sink". This solvent had two further advantages – it would disfavour the formation (or at least the survival) of the *N*(9a)-acyl linkage, and it was a superior solvent for DVB, allowing ~10 fold higher concentrations (ie, 10 mg/mL DVB). The only potential drawback was the possibility of Fmoc deprotection by pyridine, though subsequent results suggested that this was not a significant problem.

Thus, several reactions were carried out in pyridine with three equivalents of the acid chloride electrophile with or without a slight excess of TEA and with or without DMAP. The formation of compounds **2.32** (Figure 2.34), **2.33** and **2.34** (Figure 2.35) was observed (as determined by LCMS and the TFA/HPLC test outlined above), though the reactions consistently gave mixtures of compounds, including the DVB starting material and Fmoc-glycine. Furthermore, despite the use of careful and well established anhydrous techniques, reproducibility was poor.



2.33 **2.34**

Figure 2.35: Acylation products **2.33** and **2.34**.

From one reaction, involving three equivalents of the electrophile with catalytic amounts of DMAP, was isolated **2.33** in 3% yield (after multiple silica columns). **2.34** was also isolated in partial purity, which did not allow complete and unambiguous structure elucidation, but did show the key 9-NH and 2'-NH proton signals in the correct position and integrating to one proton each when examined by ^{1}H NMR spectroscopy, thus confirming that the two acyl groups (as determined by MS) were shared between both positions.

The best results obtained were from an experiment involving three equivalents of the electrophile and five equivalents of TEA in pyridine (though reproducibility problems should again be emphasised). The HPLC results from the analysis of the crude reaction mixture are given below in Figure 2.36 and Figure 2.37.

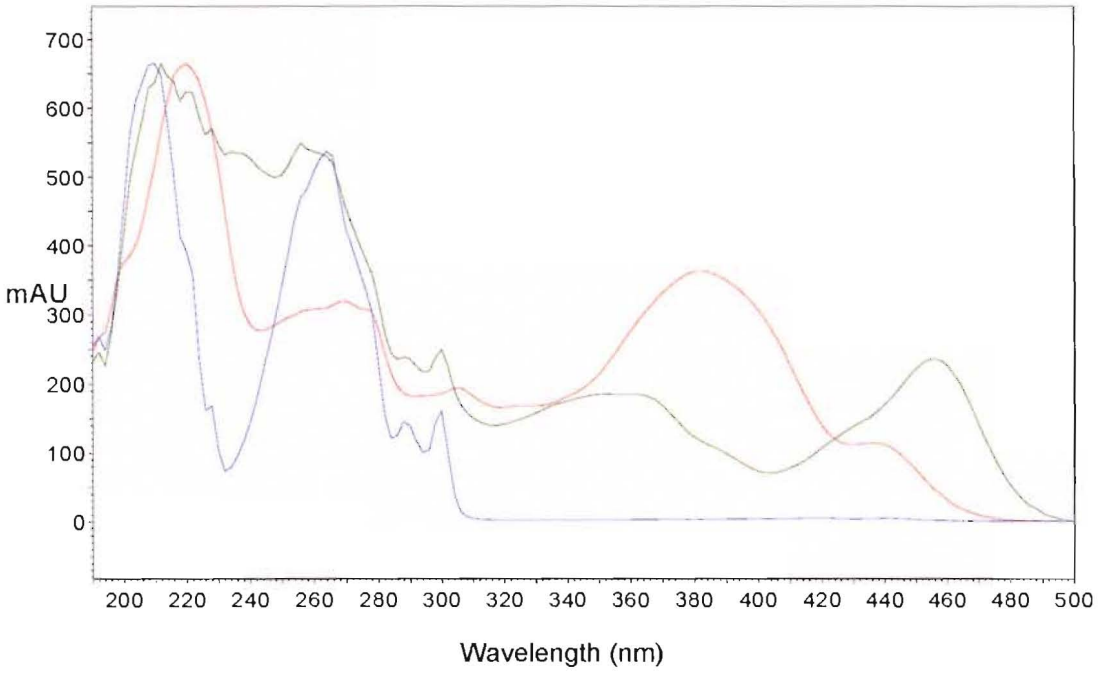


Figure 2.36: UV profiles of DVB (red), **2.33** (green) and Fmoc-glycine (blue).

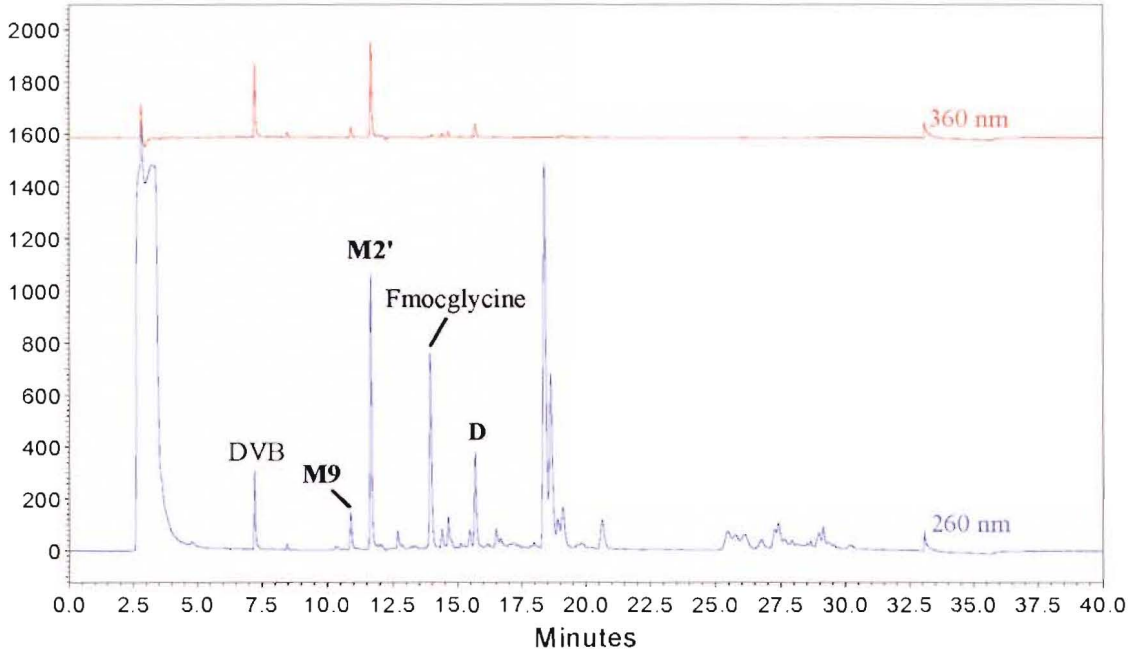


Figure 2.37: HPLC trace of a crude acetylation reaction mixture, run with the standard gradient elution protocols at 260 nm (blue) and 360 nm (red).

Figure 2.36 gives the UV profiles of DVB, Fmoc-glycine and **2.33** (the profiles are not standardised, ie, comparisons of the relative values of the extinction coefficients at any given wavelength for the three different compounds are potentially misleading). It can be seen, then, that HPLC runs examined at 260 nm will show the presence of all Fmoc- and variolin-containing compounds, while examination at 360 nm (which is the optimum wavelength when **2.32** and **2.34** are also considered) will show only the variolin material. This distinction between the two wavelengths is precisely what is shown in Figure 2.37.

It can be seen (particularly by examination of the top, red trace in Figure 2.37) that a significant amount of DVB remains unreacted (exactly how much is uncertain, as extinction coefficients for the relevant variolin compounds have not been measured). Of the DVB that did react, the N(2'a) position was the favoured site of acylation, presumably due to cleavage of most of the *N*(9a)-amide bonds that had formed. Large amounts of Fmoc-glycine were also seen. It is not known what the material eluting between 18 and 19 minutes was, other than that it contained an Fmoc chromophore; LCMS analysis performed in positive mode did not give any useful information, and attempts were not made at isolating this material. The possibility of it being an Fmoc-deprotection fulvene species is seemingly discounted by the absence of any significant amounts of Fmoc-deprotected glycine-variolin conjugates, which would be expected to elute around 6-8 minutes (the identity of the DVB peak was confirmed by LCMS).

It is possible that an equilibrium exists. It is difficult to explain why the *N*(2'a)-acylation did not go to completion. The possibility that this group became protonated as the reaction proceeded is an unlikely one, given the large amount of amine base present. Another possibility is that the pyridinium acyl salt was not a strong enough acylating agent to allow the acylation of this position. This, taken together with the possibility of an equilibrium between the pyridinium acyl salt and the variolin *N*(9a)-acylated species' (the former of which would immediately decompose following injection onto the HPLC), would explain the presence of the DVB starting material and Fmoc-glycine. However, this would also imply that any *N*(2'a)-acylation that had occurred had been carried out by the acid chloride before it had reacted with the pyridine solvent, which also seems unlikely.

Regardless of the reasons, the acylation of DVB was found to be a messy (and presumably complicated) reaction, with poor reproducibility. Factors which may affect the reaction include the possible protonation of the variolin core as the reaction proceeds, possibly modified

reactivity of either the N(9a) or N(2'a) position upon acylation of the other, and possible involvement of DVB acylation intermediates in intermolecular acyl transfers.

When it had been established that the acylation of N(2'a) of DVB would not go to completion, a crude reaction mixture, similar to that shown in Figure 2.37, was treated with piperidine in the hopes that all the variolin compounds would be converted to DVB and **2.30** (Figure 2.31). The mixture was analysed by HPLC to give a trace that was indeed much simpler than that shown in Figure 2.37. A large peak at 10.3 minutes, corresponding to the piperidine-fulvene Fmoc-deprotection adduct, was seen, as well as three variolin compounds. One of these was DVB (eluting at 7.3 minutes), while the other two eluted at 6.7 and 6.8 minutes. It is not known what these two compounds were.

It was realized at this stage that purification was going to be very difficult, given the reaction mixture containing DVB, the two unknown variolin compounds and glycine, all of which were very polar molecules. This was further complicated by the awkward solubility of the variolin compounds. An initial attempt to remove the glycine by coating C_{18} with the organic material and washing with saturated aqueous $NaHCO_3$ solution proved unsuccessful. This work was therefore abandoned in favour of more immediately promising lines of investigation (see Chapter 3), and was not revisited due to results presented in Chapter 4.

2.7 Conclusions and Future Work

The DVB synthetic protocols developed by Anderson and Morris were further refined so that DVB was synthesised on the sub-gram scale from commercially available starting materials in six steps (only three of which required chromatography) in 25% overall yield. The key developments were the application of Barbier protocols in the synthesis of triaryl acetate **2.5**, taking the crude reaction mixture from this step directly through to the next step (synthesis of core structure **2.6**), and employing DIOL-based protocols in the purification of DVB.

A convenient synthesis of ketone **2.9** was also developed, which is a potentially useful compound for the synthesis of deoxyvariolin analogues.

The 2'-thiomethyl DVB analogue **2.16** proved to be a useful compound when investigating the reaction of the 9-NH_2 group with electrophiles. These investigations were extended to DVB, where it was shown that acylation proceeds first at the N(9a) position, with anhydrides possessing sufficient electrophilicity to allow this reaction to proceed to completion. The more electrophilic acid chlorides are needed to acylate at the N(2'a) position, which is consistent with the relatively low nucleophilicity of other 2-aminopyrimidines.

The *N*(9a)-acyl linkage is stable at neutral pH but cleaved in acidic or basic environments. This would suggest that polymer therapeutics carrying DVB bound via an acyl group at the N(9a) position could give intralysosomal site-specific liberation of the cytotoxin. Synthetic routes to polymer therapeutics of this type could capitalise on the preferred acylation at the N(9a) position over the N(2'a), but would need to employ very mild synthetic conditions to preserve this delicate linkage. One possibility would be the reaction of a pHPMA-based copolymer carrying a specified stoichiometry of tetrapeptide-bearing methacryl units. Conjugation of DVB with these units using anhydride activation would probably be most suitable (EDCI has been successfully used in one such polymer therapeutic synthetic reaction).[26]

Ultimately, acylation of an *N*(9a)-protected DVB analogue is probably the preferred synthetic route to an *N*(2'a)-acylated variolin conjugate, given the messy and awkward acylation of DVB. Synthesis of **2.29** was a promising result, however alternative PMB-deprotection protocols need to be explored. Alternatively, a different protecting group could be utilised.

CHAPTER 3

DEOXYVARIOLIN ANALOGUES

3.1 Research Carried Out by Pharma Mar, SA

As was mentioned in Section 1.3.3.3, the variolin analogue production carried out in the laboratory of Dr Jonathan Morris at the University of Canterbury (UoC) was part of a larger investigation being performed at Pharma Mar, SA (a Spanish Pharmaceutical company that focuses on marine natural products). The work carried out at the University of Canterbury (UoC) is outlined in Section 3.2, while this Section gives an overview of the work carried out at Pharma Mar. It should be noted that this unpublished work is of commercial sensitivity, and so the details given here are necessarily sparse.

As part of the work carried out at Pharma Mar, around 200 analogues have been synthesised, with most being related to DVB (159) and a smaller number based on variolin B (70). Of these analogues, 35% possessed bioactivity in the 10^{-6}-10^{-7} M range, with none showing significantly better activity than variolin B or DVB. Solubility of analogues is an ongoing problem, though the stability of variolin B and DVB in aqueous solutions is very good.[89]

3.2 Research Carried Out at the UoC

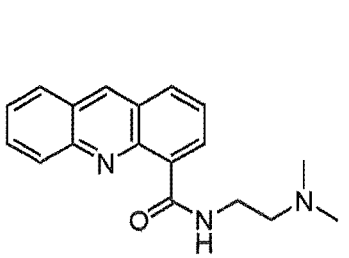
In parallel to the research being carried out at Pharma Mar, the synthetic work carried out in the group of Dr Jonathan Morris at the UoC was performed by two researchers – Dr Regan

Anderson, a PhD student at the time, followed by the author of this thesis. Dr Anderson worked primarily with the methoxy and hydroxy series (see Section 3.2.2),[87] while the work described in this thesis was involved with the deoxy series (see Section 3.2.3). Before the results are presented, however, the rationale behind the selection of the target analogues should first be given.

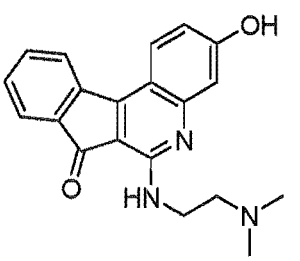
3.2.1 Rationale for target analogues

When the variolin analogue synthetic work began, it was thought likely that variolin B exerted its bioactivity through DNA intercalation (though, as is outlined in Section 1.3.2, it is now known that both variolin B and deoxyvariolin B are CDK inhibitors).[67] Attention was therefore turned towards other intercalators for clues as to the sort of structural modification that may enhance the bioactivity of the variolins.

Two such intercalators are *N*-(2-(dimethylamino)ethyl)acridine-4-carboxamide (DACA), a compound that has progressed to phase II clinical trials, and TAS-103, another antitumour agent that is also in clinical trials (Figure 3.1).[111,112] Both are dual Topoisomerase I/II poisons, stabilising the Topo-DNA complex, thus leading to accumulation of DNA strand breakages and, ultimately, apoptosis.[113-116] Both can be superficially described as planar heterocyclic compounds bearing *N,N*-dimethylaminoalkylamido/amino moieties. A crystal structure of DACA bound to a DNA oligomer shows the tertiary amine involved in an important hydrogen bond to the N(7) of a guanine residue.[113]



DACA



TAS-103

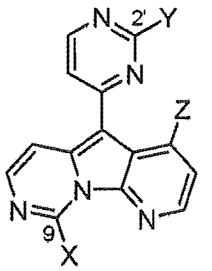
Figure 3.1: DACA and TAS-103, two DNA intercalators.

These results led to the idea that the bioactivity of variolin B may be enhanced through the inclusion of an *N,N*-dimethylaminoalkylamine moiety. Further reasons for pursuing this type of analogue was the anticipated improved solubility afforded by the basic dimethylamino moieties (which would be protonated at the physiological pH of ~7.4), and the observed improvement in nuclear localisation of small molecules bearing multiple positively charged nitrogens.[117]

3.2.2 Analogue syntheses performed prior to this thesis

As was outlined in Section 1.3.3.3, Anderson and Morris synthesised a range of variolin analogues in the methoxy and hydroxy series. Several of these analogues incorporated *N,N*-dimethylaminoalkylamines at the C(9) position, though the SAR investigation was not extended to the C(2') position. The analogues are given below in Table 3.1, along with their IC_{50} values against the P388 cell line.

It can be seen that DVB and variolin B are by far the two most bioactive analogues. The next most bioactive analogues (**3.1** and **3.2**, which are roughly an order of magnitude less bioactive than the parent compound, variolin B) bear an *N,N*-dimethylaminopropaneamine group, and so this moiety was chosen for further investigations involving the deoxy series. Analogues **3.6** and **3.7** were intermediates in the synthesis of **3.9** and **3.10**, respectively (see Section 2.3.1 for the details of the analogous synthesis in the deoxy series), and analogue **3.8** was synthesised from the bis-PMB-protected analogue (see Section 2.4.1 for details of the analogous synthesis in the deoxy series).



Compound	X	Y	Z	IC_{50} (μM)*
DVB	NH_2	NH_2	H	0.47
variolin B	NH_2	NH_2	OH	0.65
3.1	$NH(CH_2)_3NMe_2$	SMe	OMe	8.3
3.2	$NH(CH_2)_3NMe_2$	SMe	OH	10.0
3.3	NH_2	NH_2	OMe	12.1
3.4	$NH(CH_2)_2NMe_2$	SMe	OMe	26.8
3.5	$NH(CH_2)_2NMe_2$	SMe	OH	32.9
3.6	NH(PMB)	SMe	OMe	>54
3.7	NH(PMB)	SMe	OH	>56
3.8	NH(PMB)	NH_2	OH	>60
3.9	NH_2	SMe	OMe	>74
3.10	NH_2	SMe	OH	>77

* Against the P388 cell line.

Table 3.1: The methoxy- and hydroxyvariolin analogues synthesised by Anderson.

3.2.3 Analogues synthesised by the author

As has already been mentioned, the SAR work initiated by Anderson and Morris was extended to the deoxy series as part of the work described in this thesis. Synthesis of alkyldiamine analogues in the deoxy series was considered worthwhile for two reasons. Firstly, it would allow a direct comparison with the compounds synthesised by Anderson, and, secondly, it could lead to a bioactive analogue bearing a primary alkylamine that could be conjugated with the tetrapeptide

biolinker *en route* to a polymer-drug construct. This latter point became particularly relevant after shortcomings were discovered with the acylation (and stability) at the N(9a) and N(2'a) positions of the variolin core (see Sections 2.3, 2.4 and 2.5).

The deoxyvariolin analogues were synthesised according to protocols developed and employed in the methoxy- and hydroxyvariolin series by Anderson and Morris (Section 1.3.3.3, Figure 1.15, Table 3.1). These pathways are based on the difference in reactivity between the two pyrimidine C(2) positions. Specifically, the C(9) position of the key intermediate, **2.6**, is more reactive with respect to nucleophilic substitution than the C(2') position. Selective substitution by amines at C(9) of **2.6** can therefore be carried out at high temperatures (~100 °C) without any observable reaction at C(2'). Similarly, oxidation of both thiomethyl groups of **2.6** to give the bis-sulfoxide derivative, **2.25**, allows selective substitution at C(9) at room temperature in a few minutes, whereas higher temperatures (~100 °C) are needed before substitution at C(2') will proceed. The application of these reaction pathways to the deoxyvariolin series are summarised in Figure 3.2.

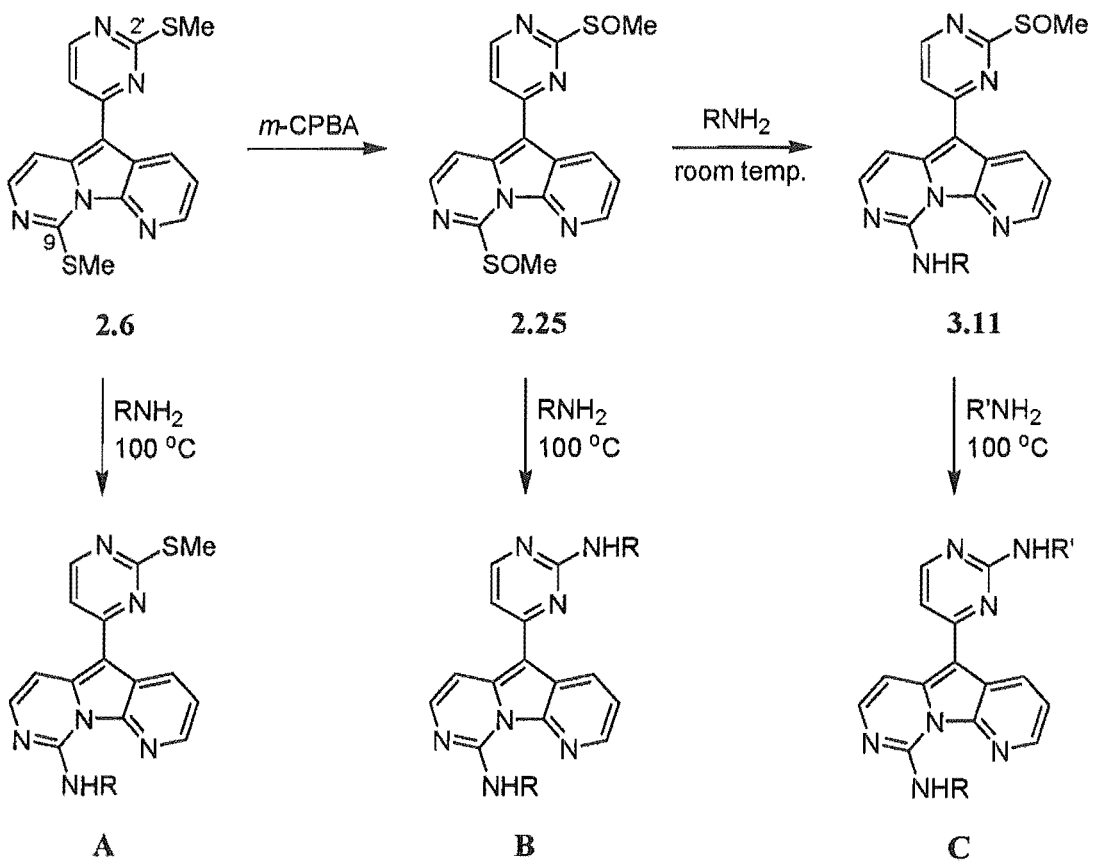


Figure 3.2: Synthetic routes to deoxyvariolin analogues.

Analogues of type **A** were therefore synthesised by heating core structure **2.6** with an excess of the nucleophilic amine. Analogues of type **B** were synthesised by first oxidising **2.6** with *m*-CPBA to give the bis-sulfoxide, **2.25**, then heating this compound with an excess of the amine nucleophile. Finally, analogues of type **C** were synthesised by reacting **2.25** with one equivalent of the first amine nucleophile at room temperature (giving substitution exclusively at the C(9)-position) to give mono-sulfoxide intermediate **3.11**, followed by the addition of an excess of the second nucleophile and heating the reaction to 100 °C. Intermediates were generally not purified.

The following Sections deal with the application of these pathways in the synthesis of twelve deoxyvariolin analogues.

3.2.3.1 Dimethylaminopropaneamine analogues

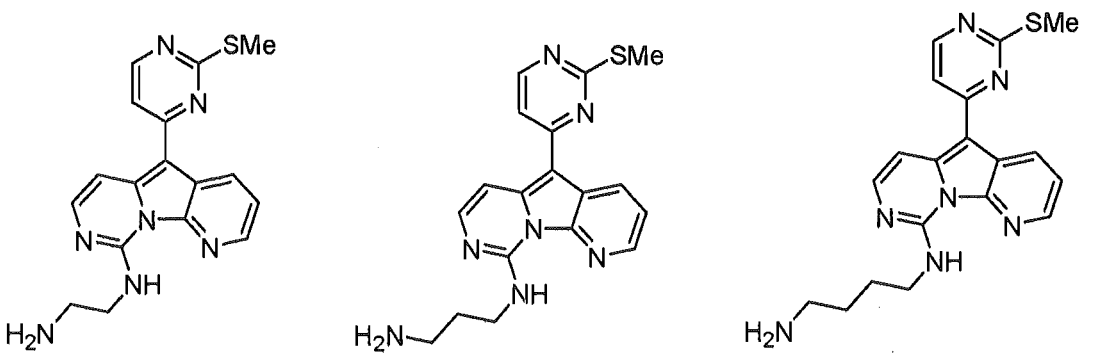
The analogues incorporating the *N,N*-dimethylaminopropaneamine moiety that were synthesised include **3.12**, **3.13**, **3.14** and **3.15**. The structures, yields and bioactivities of these compounds, along with those of their intermediates **3.16**, **3.17**, **3.18** and **3.19**, are given in Table 3.2. The syntheses proceeded without complication, with purification being carried out using reversed phase C_{18}. A discussion of the bioactivities of these analogues is given in Section 3.2.3.4.

3.2.3.2 Methoxy analogues

The 2'-methoxy analogue **3.20**, as well as the two intermediates **3.18** and **3.21** were also synthesised, using sodium methoxide as a nucleophile. See Table 3.2 for their structures, yields and bioactivities, and Section 3.2.3.4 for a brief SAR discussion.

3.2.3.3 Diamine analogues

Given the favourable bioactivities of **3.1**, **3.2** and **3.12**, taken together with the difficulties encountered in the synthesis of a variolin-tetrapeptide conjugate from analogues **2.16**, **2.27** and DVB (see Chapter 2), the analogues **3.22**, **3.23** and **3.24** (Figure 3.3) became logical targets. It was expected that these analogues would be easily conjugated with the tetrapeptide biolinker, given the presence of only one nucleophilic amine. Furthermore, and in contrast to the 9-NH_2 and 2'-NH_2 groups of DVB, the reactivity of the alkylamine moiety, and the stability of the resulting amide linkage, would be expected to be that of other simple primary amines and amides, respectively.



3.22 **3.23** **3.24**

Figure 3.3: Synthetic targets for incorporation into polymer-drug conjugates.

Initial attempts at synthesising these analogues were performed in parallel, with the nucleophilic amines being used straight from the bottle without any attempts made to dry the reagents first. This casual experimental technique was due to the fact that water contamination had not presented itself as a problem with *N,N*-dimethylaminopropaneamine as the nucleophile. In the reactions involving the diaminoalkanes, however, water contamination led to the isolation of the degradation product **3.25** (Figure 3.4) from the reaction mixtures. A proposed mechanism of formation of **3.25** is given in Figure 3.4. The key steps in the decomposition are the two successive imine hydrolyses, followed by a high-temperature decarbonylation reaction. The extent of formation of **3.25** varied between the three reactions, with degradation in the ethylene- and propylenediamine systems being complete after 20 h at 100 °C, and that in the butanediamine system being only partial (~40% under the same reaction conditions). This difference may reflect the more favourable formation of 5- and 6-membered rings over the 7-membered ring, respectively, during the decomposition. It may also reflect the different amounts of water contamination in each of the three nucleophilic reagents (1,2-diaminoethane and 1,3-diaminopropane are liquids at room temperature, whereas 1,4-diamino butane is a crystalline solid). **3.25** possessed an IC_{50} of 35 μM against the P388 cell line.

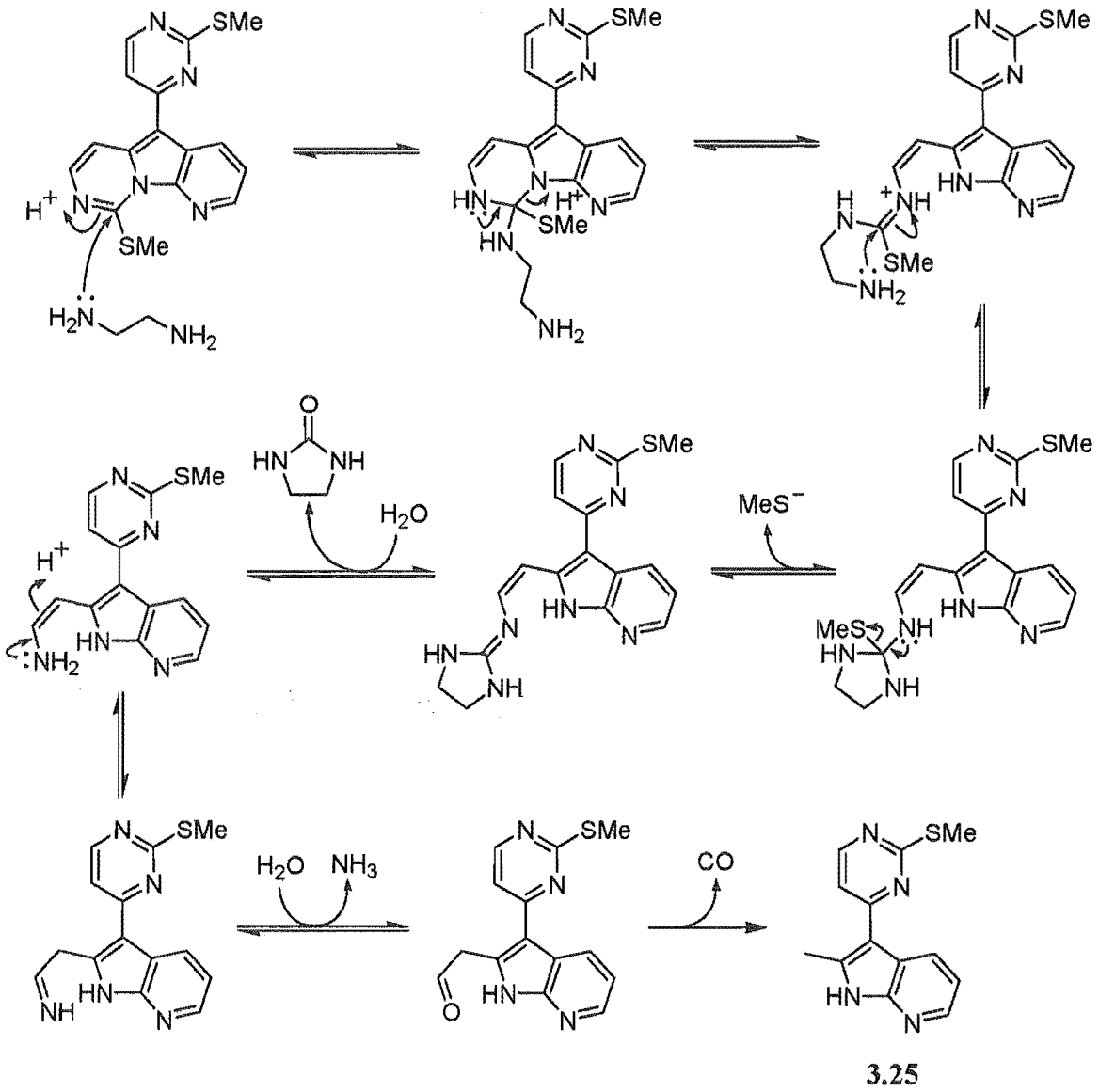


Figure 3.4: Proposed mechanism of formation of **3.25**.

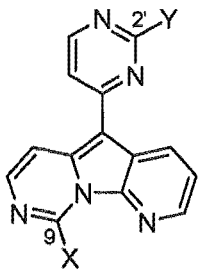
Given the difficulty in handling polar compounds such as **3.22**, **3.23** and **3.24**, it was necessary to rigorously exclude water from the reaction mixtures in order to avoid any formation of **3.25** and thus greatly simplify the workup/purification process. The propylene- and butylenediamines were dried by azeotropic distillation using toluene in a Dean-Stark apparatus. The ethylenediamine was not dried successfully by this methodology, probably because its boiling point (118 °C) exceedsd that of toluene by only 7 °C. All three amines were also converted to their hydrochloride salts and recrystallised from H_2O/EtOH. Conversion of the salts back to the free bases, and isolation of the anhydrous free bases, however, was difficult, and so this methodology was abandoned. Due to the difficulty in obtaining 1,2-diaminoethane as an anhydrous solution, the synthesis of **3.22** was not pursued. Moreover, results obtained by

Anderson and Morris in the hydroxy and methoxy series suggested that this analogue would show inferior bioactivity to **3.23** (see compounds **3.1**, **3.2**, **3.4**, and **3.5**, Table 3.1).

Using dry toluene solutions of 1,3-diaminopropane and 1,4-diaminobutane, analogues **3.23** and **3.24** were successfully synthesised without any discernable decomposition (based on ^{1}H NMR spectroscopic analysis of the crude reaction mixtures). A large excess of the nucleophile was used to avoid dimer formation. A 15-fold excess was initially employed, but that was subsequently reduced to 5-fold in the diaminopropane reaction without any observable dimer formation. Purification was difficult due to the high polarity of both compounds (with **3.23** and **3.24** being moderately water soluble). Both LH20 and DIOL chromatography were employed with limited success. Kugelrhor distillation at 120 °C, 0.03 mm Hg allowed the efficient removal of any unreacted nucleophile with no observable decomposition of the variolin product. This allowed both analogues to be produced in high purity in near-quantitative yield. Further experimentation led to the discovery that **3.23** could be isolated in high yield and purity by washing a solution of the analogue in dichloromethane with saturated aqueous brine solutions (the solubility of **3.23** in saturated aqueous brine solution is poor).

3.2.3.4 Analogue bioactivities

The bioactivity and yield data for the twelve analogues whose syntheses are briefly discussed above, along with the six analogues (DVB, **2.6**, **2.16**, **2.17**, **2.26** and **2.27**) whose syntheses were discussed in chapter 2, are summarised in Table 3.2.



Compound	X	Y	IC_{50} (μM)*	% Yield (# of steps)**
DVB	NH_2	NH_2	0.47	63 (3)
3.19	NH(PMB)	$NH(CH_2)_3NMe_2$	2.1	68 (3)
3.15	NH_2	$NH(CH_2)_3NMe_2$	2.5	22 (4)
3.20	NH_2	OMe	3.6	18 (4)
2.16	NH_2	SMe	3.9	93 (2)
2.27	NH(PMB)	NH_2	5.4	60 (3)
3.23	$NH(CH_2)_3NH_2$	SMe	5.7	94 (1)
3.13	$NH(CH_2)_3NMe_2$	$NH(CH_2)_3NMe_2$	6.0	63 (2)
3.17	$NH(CH_2)_3NMe_2$	NH(PMB)	8.3	63 (3)
3.12	$NH(CH_2)_3NMe_2$	SMe	11	98 (1)
3.14	$NH(CH_2)_3NMe_2$	NH_2	17	38 (4)
3.24	$NH(CH_2)_4NH_2$	SMe	21	99 (1)
3.18	NH(PMB)	SOMe	>28	63 (2)
3.16	$NH(CH_2)_3NMe_2$	SOMe	>28	Not taken
2.17	NH(PMB)	SMe	>29	99 (1)
3.21	NH(PMB)	OMe	>30	61 (3)
2.26	NH(PMB)	NH(PMB)	>34	72 (2)
2.6	SMe	SMe	>37	NA

* Against the P388 cell line.

** The yield and number of steps are given from the key intermediate **2.6**.

Table 3.2: A range of deoxyvariolin analogues and their bioactivities.

The immediate conclusion that can be drawn from these SAR results is that all the modifications at C(9) and/or C(2') of DVB that were investigated led to at least a five-fold decrease in bioactivity, with all but two of the synthetic intermediates showing a decrease in bioactivity of at least two orders of magnitude. Surprisingly, the next most bioactive analogue after DVB was **3.19**. There are no obvious patterns or general structure-activity relationships that can be inferred from the results, with one result seemingly contradicting a conclusion that might be drawn from another. Also, an error margin of roughly a factor of two is present in the bioassay, so small differences in bioactivity should be discounted.

The fact that the P388 cell line was the only one investigated represents a further shortcoming of these results, as is evidenced in the results obtained by Pharma Mar, SA in their SAR work with the variolins. In their 2003 patent,[91] for example, they report the bioactivity of a range of around 100 different analogues against up to 17 different cell lines. In short, the cytotoxicities of a single analogue often vary significantly between different cell lines, sometimes in excess of an order of magnitude, and different cell lines show different degrees of relative sensitivity to different analogues.

Furthermore, while bioassays such as the P388 *in vitro* assay are invaluable in SAR work such as this, they do possess some important shortcomings, particularly with respect to this project. The assay conditions are necessarily contrived, and so do not accurately mimic *in vivo* human physiology (where pharmacokinetics and pharmacological profiles become important). This alone may affect the results significantly. However, in this investigation a more serious shortcoming exists: namely, that the cytotoxins presented in Table 3.2 were submitted to the bioassay as the free drugs, whereas the aim of this project is to incorporate cytotoxins into polymer-drug constructs. As was discussed in Section 1.2, this will drastically alter the mechanism of drug delivery to the intracellular site of action, which could in turn affect the relative bioactivities of the deoxyvariolin analogues.

Despite these shortcomings of the bioassay, however, the information is still useful and strongly suggestive. An important result was that analogue **3.23** possessed reasonable bioactivity (more so than **3.24**), and so it was chosen as a suitable drug for development into a polymer-drug construct. Though the bioactivity was less than what is generally accepted as being required of anticancer drugs (nanomolar levels), the tumour-targeting and improved pharmacological profile afforded by conjugation onto a polymer should offset this relatively low bioactivity when used *in vivo*.

It is noteworthy that this analogue was synthesised from commercially available starting materials in 4 steps (with only one chromatographic step) in 38% overall yield.

3.3 Acylation of **3.23**

As discussed in Section 1.2.3.2, the polymer-drug constructs that were the ultimate goal of this work were to be synthesised by the reaction of pMAOS with appropriate nucleophiles, which included tetrapeptide-drug constructs. Conjugate **3.26** was therefore an immediate synthetic target (Figure 3.5).

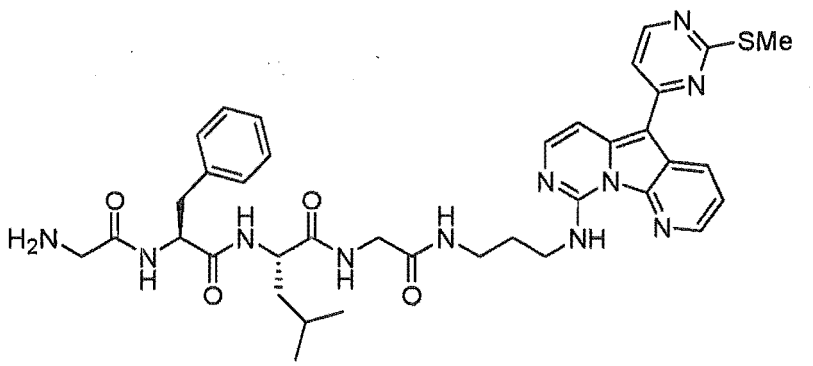


Figure 3.5: Conjugate **3.26**.

An EDCI-mediated coupling between FmocGFLG and **3.23** gave the protected conjugate, **3.27** (Figure 3.6). DIOL chromatography allowed the efficient purification of this compound, whereas LH20 chromotography led to unsatisfactory results.

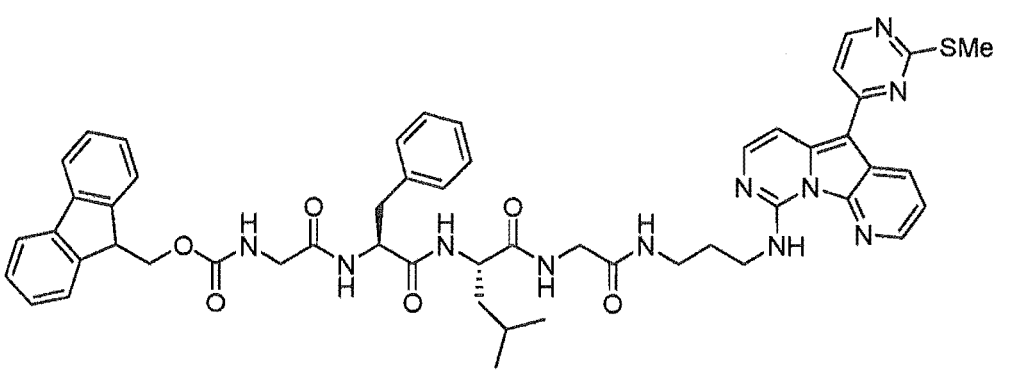
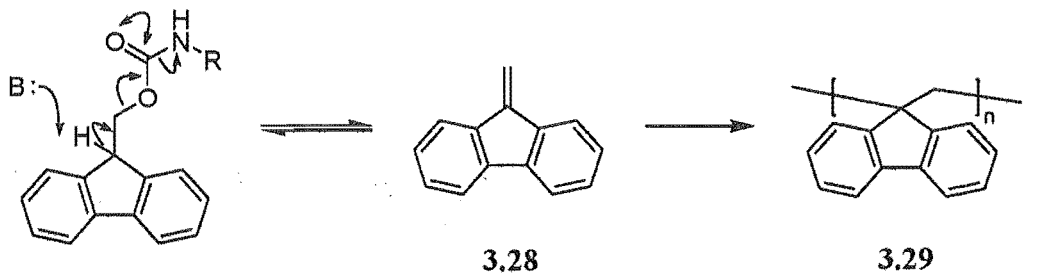


Figure 3.6: Conjugate **3.27**.

Attempts at removing traces of solvent from the sample by heating under reduced pressure (75 °C, 0.03 mm Hg, 20 h) led to decomposition of **3.27** to **3.26**, with the concomitant formation of a material that showed low solubility in all solvents investigated, including MeOH, DMSO, THF, CH_2Cl_2 and hexanes. Given its low solubility, this material was unable to be characterised, though is presumed to be the polymer **3.29**, formed from the dibenzofulvene **3.28**, which is in turn the product of Fmoc deprotection (Figure 3.7). The unpredictable polymerisation of **3.28** is well known.[98] FmocGFLG had previously been seen to be stable under the conditions that led to the decomposition here, and so intermolecular catalytic participation by the basic variolin heterocyclic core is proposed to be the reason for the observed decomposition.



3.28 **3.29**

Figure 3.7: Formation of **3.29**.

The mixture of desired product and presumed polymer was taken directly through to the Fmoc-deprotection step. Deprotection was carried out in a 2.5% piperidine/THF solution. Addition of hexanes led to precipitation of the desired product, which was isolated by filtration to give the desired product with a small amount of contamination by **3.29**. This mixture was then dissolved in THF and filtered to remove the polymeric contaminant. The deprotection step was presumed to be quantitative, with the desired product **3.26** being obtained in 62% yield over 2 steps (that is, from **3.23**). The product was of sufficiently high purity (see Figure 3.8) to negate the need for any further purification. This was fortunate, as it would be difficult to purify **3.26** by chromatography (laborious reversed phase preparative HPLC would almost certainly have been required).

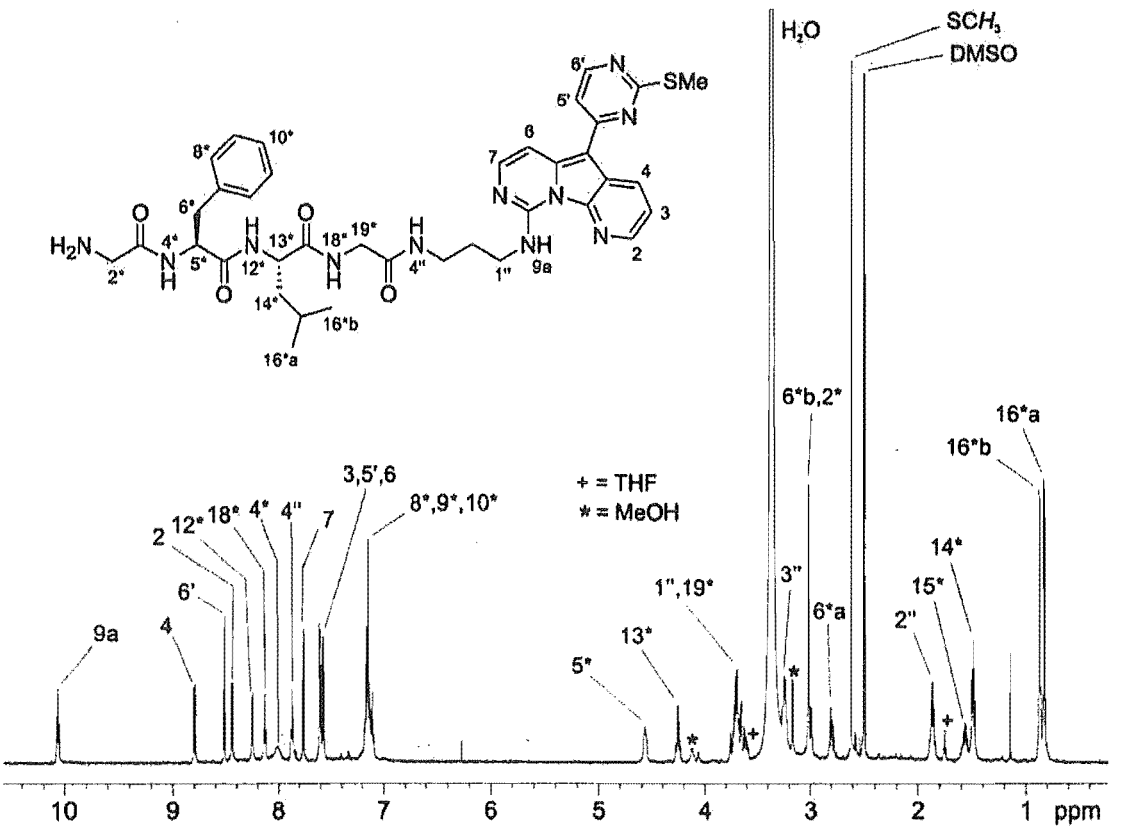
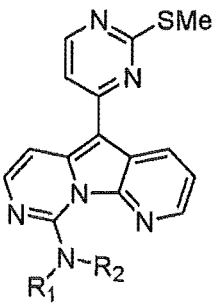


Figure 3.8: ^{1}H NMR spectrum of **3.26** in d_6-DMSO

The bioactivities of **3.27** and **3.26** are very similar (see Table 3.3), and retain over half the bioactivity of the parent compound, analogue **3.23**. This is less than the error margin for the bioassay, which is considered to be ~2-fold. This result suggests either that the conjugate itself is appreciably bioactive, or that the tetrapeptide motif is being efficiently cleaved intralysosomally by cathepsin proteases. While further investigations are required to determine which of the two is correct, the former explanation is the most likely.

Table 3.3 gives the structures and bioactivities of some of the acylated variolin compounds that were synthesised as part of the work described in this chapter and in Chapter 2.



Compound	R_1	R_2	IC_{50} (μM)*
3.27	$(CH_2)_3$NHGLFGFmoc	H	9.0
3.26	$(CH_2)_3$NHGLFG	H	9.9
2.19	Ac	H	12.6
2.20	Ac	Ac	16.1
2.24	CO(*t*-Bu)	H	>8.0
2.18	GLFGFmoc	H	>13.8

* Against the p388 cell line

Table 3.3: Bioactivities of some acylated deoxyvariolins.

Interestingly, conjugate **2.18** (whose synthesis is described in Section 2.3.3.1) is less bioactive than conjugate **3.27**, despite analogue **2.16** being more bioactive (IC_{50} = 3.9 μM) than analogue **3.23** (IC_{50} = 5.7 μM). This may simply reflect the aforementioned error margin, though the exact bioactivity of **2.18** is not known. By virtue of its larger size and higher polarity, conjugate **2.18** would most likely enter the cells via the lysosomal pathway. It has already been postulated (see Section 2.3.5) that the lysosomal environment would facilitate the degradation of **2.18** to **2.16**, though the observed *in vitro* bioactivity of this compound would suggest otherwise. Analogues **2.19** and **2.24** (and, to a lesser extent, **2.20**) are related compounds, in that they are derived from **2.16** by acylation at N(9a). These small molecules, however, are most likely transported directly across the cell membrane in the *in vitro* bioassay. Given the lability of the *N*(9a)-amide bond (discussed in Section 2.3.5), it is unclear whether the bioactive agents are the compounds themselves, or the degradation product **2.16**. More data are necessary to determine why the bioactivity of **2.18** is so much lower than that of **2.16**, and how it compares with the bioactivities of **2.19**, **2.20** and **2.24**.

With **3.26** in hand, and demonstrable bioactivity of this conjugate *in vitro*, attention was focussed on the incorporation of this compound into a polymer-drug construct. Efforts toward this goal are described in the following chapter.

3.4 Conclusions

The synthetic protocols developed by Anderson and Morris were successfully employed in this work in the synthesis of thirteen deoxyvariolin analogues from the key intermediate **2.6**. Substitution by amines at C(9) of **2.6** gave the desired products in near quantitative yield. Syntheses which first required the oxidation of **2.6** to **2.25** proceeded in 60-65% yield, while PMB-deprotection reactions give yields of 30-50% (perhaps reflecting, at least in part, the small scale that these reactions were often carried out on).

All analogues synthesised displayed inferior bioactivity to DVB in the P388 *in vitro* bioassay. There were no obvious structure-activity relationships, with steric bulk at both positions being tolerated to different degrees between different analogues. Both primary amines of DVB appear to be required for sub-micromolar bioactivity. The inclusion of *N,N*-dimethylaminopropane-amine moieties affected the bioactivity adversely, and in a comparable manner to the methoxy and thiomethoxy moieties at the C(2') position (with the surprising exception of analogue **3.19**), and to the amino, *p*-methoxybenzylamine and alkyldiamine moieties at the C(9) position. It is now known that DVB is not an intercalator, and so incorporation of these moieties in hindsight would not necessarily be expected to improve the bioactivity.

Analogue **3.23**, a synthetically accessible DVB analogue incorporating a diaminopropane moiety, was approximately an order of magnitude less bioactive than DVB. While its bioactivity was relatively low, the improved pharmacokinetics and pharmacological profile afforded by polymer therapeutics made this analogue a reasonable candidate for incorporation into a polymer-drug conjugate. Given the presence of a single aliphatic amine, conjugation of **3.23** with the tetrapeptide biolinker proceeded without complication. The conjugate, **3.27**, could be easily purified by DIOL chromatography, which eliminated the need for chromatographic purification of **3.26**, the Fmoc-deprotected conjugate.

CHAPTER 4

POLYMER CHEMISTRY

4.1 Introduction

With conjugate **3.26** in hand, efforts towards the synthesis of a polymer therapeutic via the Godwin and Brocchini protocols began. The first step was the synthesis of a sample of pMAOS possessing suitable molecular weight characteristics. Before this is described, however, some important background information regarding the measurement and quantification of the molar mass distribution within a polymer sample will be given.

4.1.1 Polydispersity (PD)

As was outlined in Section 1.2.2.3, the improved pharmacokinetics afforded by polymer therapeutics can be attributed to their molecular size. It is important to consider, then, not just the average molar mass of a polymer sample, but also the molar mass distribution within that sample. The breadth of this distribution can be approximated and represented by a single value - the *polydispersity* (PD) of a sample.

PD will be defined shortly, but first a brief summary of the methods used in the measurement of the molar mass characteristics of a polymer sample is needed. Analysis of polymer samples by the size exclusion-based technique Gel Permeation Chromatography (GPC) will produce a curve that gives the mass fraction of the sample, w_i, at any given molar mass, M_i (it should be noted that what is actually measured is the molecular size, which may vary between different types of

polymers of the same molar mass). In order to describe the general characteristics of such a curve, at least in terms of its breadth and height, a PD value can be assigned. This PD value is a function of two types of molar mass averages, defined below.

The *number-average molar mass* ($\bar{M}_n$) is defined[118] as 'the sum of the products of the molar mass of each fraction multiplied by its mole fraction', ie,

$$\bar{M}_n = \sum X_i M_i$$

where X_i is the mole fraction of molecules of molar mass M_i and is given by the ratio of N_i (the number of molecules in a discrete size fraction i) to the total number of molecules.

The *weight-average molar mass* ($\bar{M}_w$) is defined[118] as 'the sum of the products of the molar mass of each fraction multiplied by its weight fraction', ie,

$$\bar{M}_w = \sum w_i M_i$$

where w_i is the mass of molecules of molar mass M_i divided by the total mass of the sample.

Having obtained the above values for a given sample by GPC analysis, the PD of that sample is calculated by dividing the weight-average molar mass by the number-average molar mass, ie,

$$\text{PD} = \bar{M}_w / \bar{M}_n$$

Thus, a polymer sample comprised of molecules of uniform molar mass, or one showing *monodispersity*, will have PD = 1.0. Most polymer samples, however, will be comprised of molecules of varying sizes, and as this breadth of molar mass distribution increases, so too will the PD value.

It should be noted that the interpretation of GPC traces to give PD values is somewhat subjective, and so different researchers may arrive at different PD values for similar polymer samples. Furthermore, no rigorous investigation into the relationship between PD and therapeutic efficiency has been carried out. As a guideline, however, a PD of < 1.3 was considered desirable for the first generation of polymer therapeutics of type **1.1** that were the target of this study, which would ensure that bioactivity would be closely related to the $\bar{M}_n$.

4.2 pMAOS Synthesis

Having decided that pMAOS would be employed as the activated polymeric precursor, it was necessary to synthesise a sample of pMAOS of low PD. pMAOS is synthesised by the polymerisation of methacryloxy succinimide, as shown in Figure 4.1.

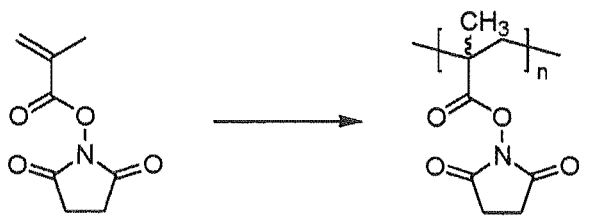


Figure 4.1: Synthesis of pMAOS from MAOS.

The main challenge presented by this reaction, then, was to control the polymerisation process to give a polymeric product of low PD.

4.2.1 Atom Transfer Radical Polymerisation (ATRP)

4.2.1.1 The literature account

As was outlined in Sections 1.2.3.2, Godwin *et al.* reported that controlled polymerisation protocols (specifically, ATRP, outlined below) can be employed in the synthesis of pMAOS to give polymer samples of low PD.[60] The investigators reported a range of syntheses of pMAOS under differing conditions, one of which gave a product with $\bar{M}_n$ = 29 kDa and PD = 1.14. Similar results were reported by Monge and Haddleton.[62] In terms of PD values, subsequent work toward the synthesis of pMAOS via other types of controlled polymerisation did not improve on this result,[119] and so it was decided that these protocols would be employed in this work.

The copper-mediated ATRP protocols (Figure 4.2) that were employed utilised an alkyl bromide initiator and copper bromide/2,2'-bipyridine complex 'catalyst'. The catalyst cycles between the Cu(I) and Cu(II) oxidation states, with bromide concomitantly being associated with either the growing polymer chain or the inorganic copper salt, respectively. In this way, the growing

polymer chain spends only a fraction of the time as a reactive free radical. This effectively lowers the concentration of this species relative to the acrylamide monomer, which ultimately will favour the polymerisation reaction over competing chain termination events (which occur when two growing polymer chains react with one another).

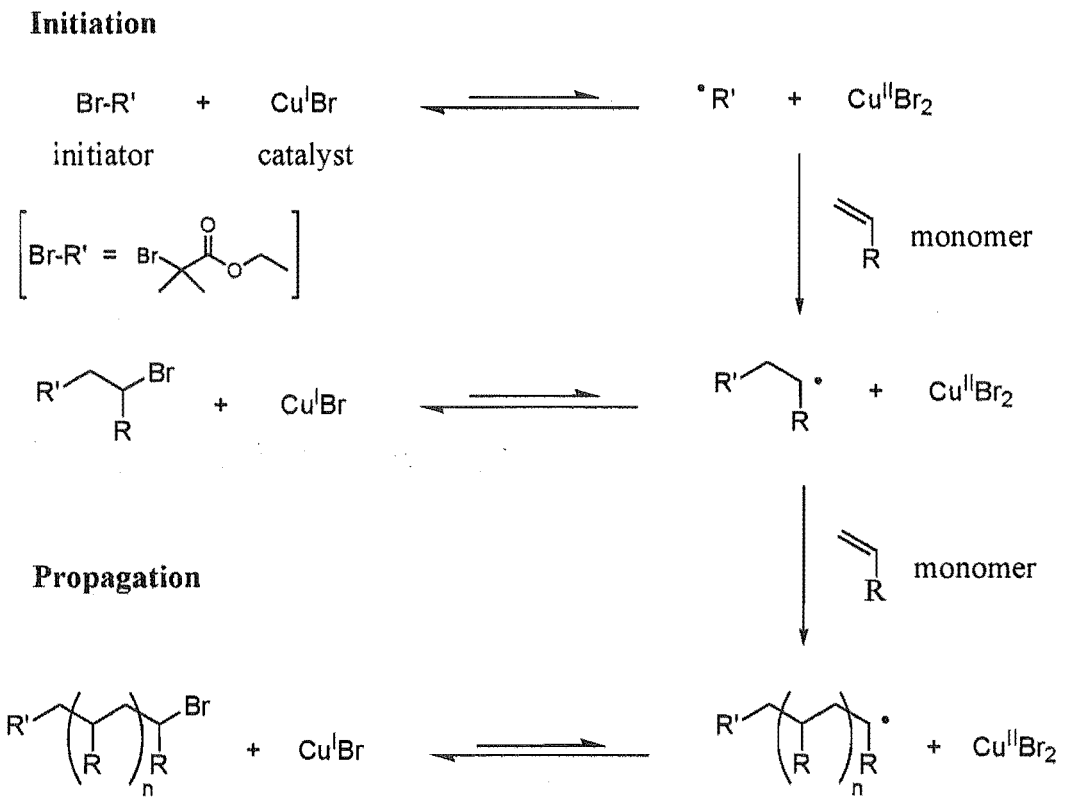


Figure 4.2: The mechanism of the copper-based ATRP system.

4.2.1.2 Experimental results

MAOS was synthesised via a DCC coupling between methacrylic acid and NHS to give the activated ester monomer, free of DCU. This monomer was polymerised by ATRP under protocols similar to those reported[60] to give pMAOS, which was then analysed by GPC. A summary of the GPC results and comparison with those from the literature is given in Table 4.1.

Experiment	Yield (%)	M_{target}[a]	$\bar{M}_n$ (gmol^{-1})	$(\bar{M}_w/\bar{M}_n)$
Literature[60]	96	17 600	29 000	1.14
Observed	66	13 400	24 900	1.87

[a] Target molecular weight calculated by $M_{target} = ([m]_0/[i]_0) \times Mr_m \times$ yield, where $[m]_0$ = initial concentration of the monomer, $[i]_0$ = initial concentration of the initiator and Mr_m = the MW of the monomer.

Table 4.1: Published and observed results for the synthesis of pMAOS by ATRP.

The difference between the two sets of results is pronounced, with the PD of the sample synthesised in this work being much higher than in the published results. There were a few differences between the published protocols and those employed here: the initiator used in the literature was 2-hydroxyethyl 2-bromoisobutyrate, whereas ethyl 2-bromoisobutyrate was used in this work; an initiator:catalyst:monomer ratio of 1:1:100 was reported in the literature, whereas the work described here used a ratio of 1:1:110, and the literature report specified a reaction time and temperature of 10-15 minutes at 100 °C, while reaction conditions of 90 °C for four hours were used here. While these discrepancies could be expected to give some deviation from the reported results, they would not account for the large difference in PD values. In fact, lower temperatures have been reported to improve the polymerization process for this particular system.[62]

These ATRP protocols were explored in more detail in a separate investigation carried out by Uma Adash. In her investigation, eleven separate syntheses of pMAOS by ATRP under differing conditions were carried out; the lowest PD obtained in this investigation was 1.68, with most values being close to or exceeding 2.

In light of these results, other controlled radical polymerisation protocols were investigated.

4.2.2 RAFT synthesis

In a parallel study, and as an alternative to ATRP, Adash investigated controlled radical polymerisation of MAOS by Reversible Addition-Fragmentation chain Transfer polymerisation

(RAFT). This investigation yielded more favourable results, and so these protocols were utilised as part of this work.

4.2.2.1 Background information

RAFT is the same in principle as ATRP insofar as the growing polymer chain spends only a fraction of the time as a free radical. RAFT, however, employs dithioacids in the place of the metal-based system used in ATRP. In a typical RAFT synthesis AIBN is used as initiator and a dithioacid derivative, or *RAFT agent*, 'caps' the growing polymer chain in an equilibrium reaction favouring the capped chain over the free radical. The RAFT agent employed in this work was 2-(2-cyanopropyl)dithiobenzoate, **4.1** (Figure 4.3).

As is shown below in Figure 4.3, not just the AIBN initiator but also the RAFT agent itself will initiate polymerisation reactions. It is therefore the combined stoichiometries of the initiator and the RAFT agent (which is usually present in a high excess relative to the initiator) which determine the number of initiation events, and therefore the M_{target}. It should also be pointed out that, though not shown in the diagram, the carbon-centred radical species is also able to participate in chain-elongation polymerisation reactions, and will do so to a greater extent as the RAFT agent is consumed by initiation events.

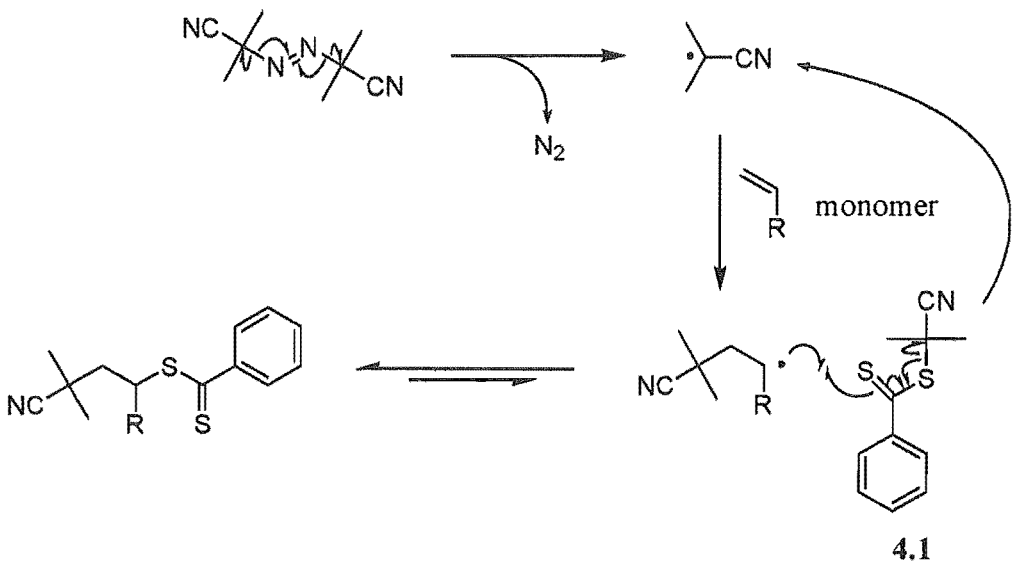


Figure 4.3: The mechanisms of initiation in a RAFT polymerisation.

The number of free radicals within the system at any one time is determined by the concentration of the initiator. Upon completion of all the initiation events, these free radicals will be centred on either growing polymer chains or a trigonal intermediate, **4.2**, incorporating the RAFT agent and two growing polymer chains (Figure 4.4).

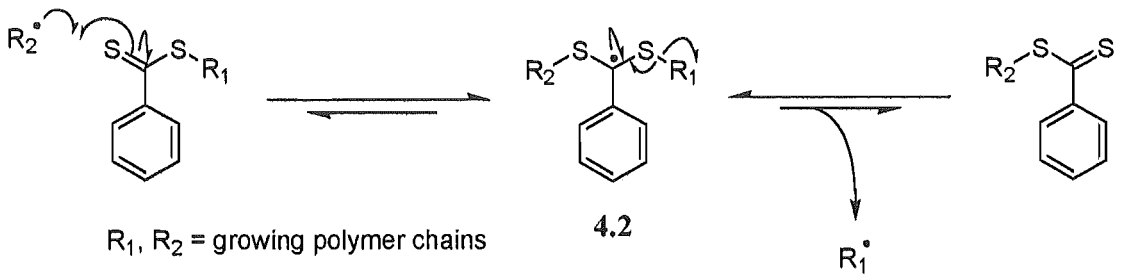


Figure 4.4: The equilibrium between free radical species during a RAFT synthesis.

Obviously, chain elongation can only proceed when a free radical is centred on a growing polymer chain. From this it follows that a highly controlled polymerisation will be one where the equilibrium reactions shown above are highly facile, with the equilibrium concentrations strongly favouring the trigonal intermediate **4.2**.

It has been reported that RAFT protocols may be employed in the synthesis of pMAOS with PD<1.3.[120,121]

4.2.2.2 Experimental results

pMAOS synthesised by Adash via the protocols of Schilli *et al.*,[120] using a 1:57 ratio of RAFT agent to monomer, possessed a much more favourable molecular weight profile (see Table 4.2) than that synthesised by ATRP.

Experiment	M_{target}[a]	Yield (%)	$\bar{M}_n$ (gmol^{-1})	($\bar{M}_w/\bar{M}_n$)
RAFT	2 600	90	30 000	1.36

[a] Target molecular weight calculated by M_{target} = $\{[m]_0/([i]_0+[r]_0)\} \times Mr_m \times$ yield; where $[m]_0$ = initial concentration of the monomer, $[i]_0$ = initial concentration of the initiator, $[r]_0$ = initial concentration of the RAFT agent and Mr_m = the MW of the monomer.

Table 4.2: Molecular weight data of a pMAOS sample synthesised by RAFT.

While the observed $\bar{M}_n$ was far from the M_{target}, the average molecular weight of 30 kDa and PD value of 1.36 were considered acceptable (though the PD was less than ideal) for the use of this polymer in the synthesis of a first generation of polymer therapeutics. The synthesis was therefore repeated to obtain sufficient quantities of polymer to proceed to the next stage of investigations, namely, polymer therapeutic syntheses.

4.3 Reactivity of pMAOS

4.3.1.1 Application of the literature protocols

The following reaction (Figure 4.5) was attempted. The copolymer intermediate was not isolated, rather it was formed and reacted *in situ*, in accordance with the reported protocols.

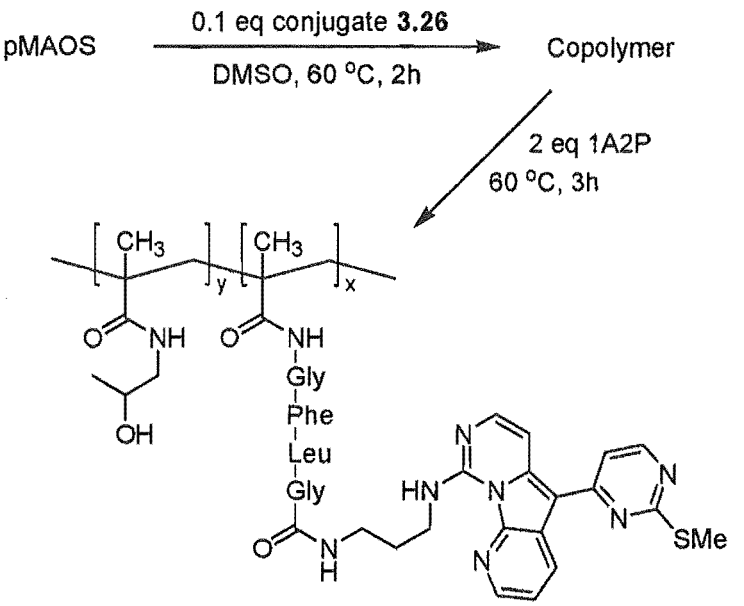


Figure 4.5: The attempted synthesis of a variolin-containing polymer therapeutic.

The reaction mixture was purified directly by LH20 size exclusion chromatography, during which time the yellow colour (characteristic of variolins) was seen associated with both the high and low molecular weight fractions. In all, seven fractions were collected, concentrated and analysed by a combination of ^{1}H NMR spectroscopy and MS(ESI).

Disappointingly, the yellow polymeric material was shown by ^{1}H NMR spectroscopy (by comparing the variolin N(9a)*H* signal with the various polymer signals) to contain less than 2 mol% variolin material, rather than the expected 10 mol%. Of the remaining material, one of the late-eluting, low Mr fractions was shown by NMR and MS to be reasonably pure conjugate **3.26**, though the amount recovered was estimated to be only a fraction of that used in the reaction. The remaining fractions were mixtures of low Mr compounds, some of which contained unidentified variolin material that was not conjugate **3.26**.

The fact that only a fraction of the variolin conjugate had ended up on the polymer was surprising, especially given that a reaction time and temperature in the first step exceeding the literature protocol by 45 minutes and 10 °C, respectively, had been used.* The low recovery of unreacted conjugate **3.26** indicated that this material was probably being consumed through a competing side reaction(s). Heating a DMSO solution of the conjugate under the same reaction conditions but in the absence of polymer led to no decomposition, suggesting that the polymer was participating in the side reactions.

Further attempts at the synthesis of a polymer therapeutic via these protocols all gave similar results. Furthermore, isolation and NMR analysis of the polymer intermediate in these reactions showed an unidentified broad polymeric peak at ~2.5 ppm, and NMR analysis of the polymeric products following the second step consistently gave polymer backbone signals integrating higher than expected relative to the polymer side-chain 1A2P signals (an explanation of these results is given below in Section 4.3.2). It was therefore decided that the two-step reaction would be further investigated with a model system, using only 1A2P as the nucleophile.

4.3.2 Reaction of pMAOS with 1A2P

(The work described in this Section was carried out in collaboration with Dr Sean Devenish. Dr Devenish's contributions are indicated wherever relevant.)

Investigations involving the reaction of pMAOS with 1A2P revealed two unexpected side reactions (see Sections 4.3.2.1 and 4.3.2.5) that, taken together, explain the failure of the

* The literature[60] protocols used conditions of 50 °C and 1.25 h, while this experiment was run at 60 °C for 2 h.

polymer therapeutic synthetic attempts, and explain the anomalies seen in the NMR spectra of the polymeric intermediates and products.

4.3.2.1 Ring-opening of the succinimide residues

Reactions of pMAOS with 1A2P at differing temperatures consistently gave a polymeric product which, when analysed by ^{1}H NMR spectroscopy, displayed the same non-pHPMA polymer peak at ~2.5 ppm (specifically, 2.4 ppm in d_6-DMSO (see Figure 4.9) and 2.6 ppm in d_4-methanol) that was mentioned above. All other signals were in the correct positions for a pure sample of pHPMA.

The identity of this signal at ~2.5 ppm was revealed through 2D NMR experiments, which were performed by Dr Devenish. An HSQC-DEPT experiment indicated that this peak corresponded to a pair of methylenes with carbon chemical shifts of 28.2 and 30.8 ppm (Figure 4.6). In a CIGAR experiment, correlations were observed from 2.6 ppm to two carbonyl resonances at 170.3 and 173.5 ppm (see Figure 4.7; the two ^{13}C correlations at 80.3 and 83.5 are fold-backs from 170.3 and 173.5 ppm, respectively).

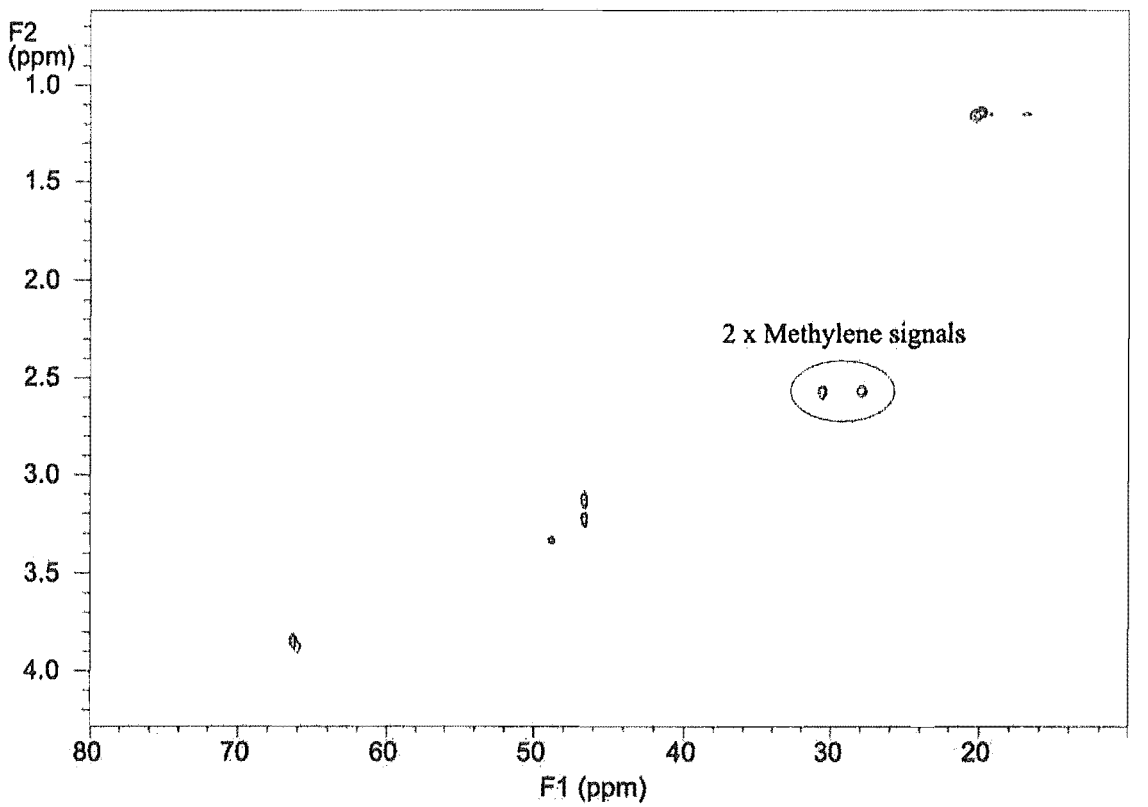


Figure 4.6: HSQC-DEPT NMR spectrum, taken in CD_3OD, of copolymer **4.3**.

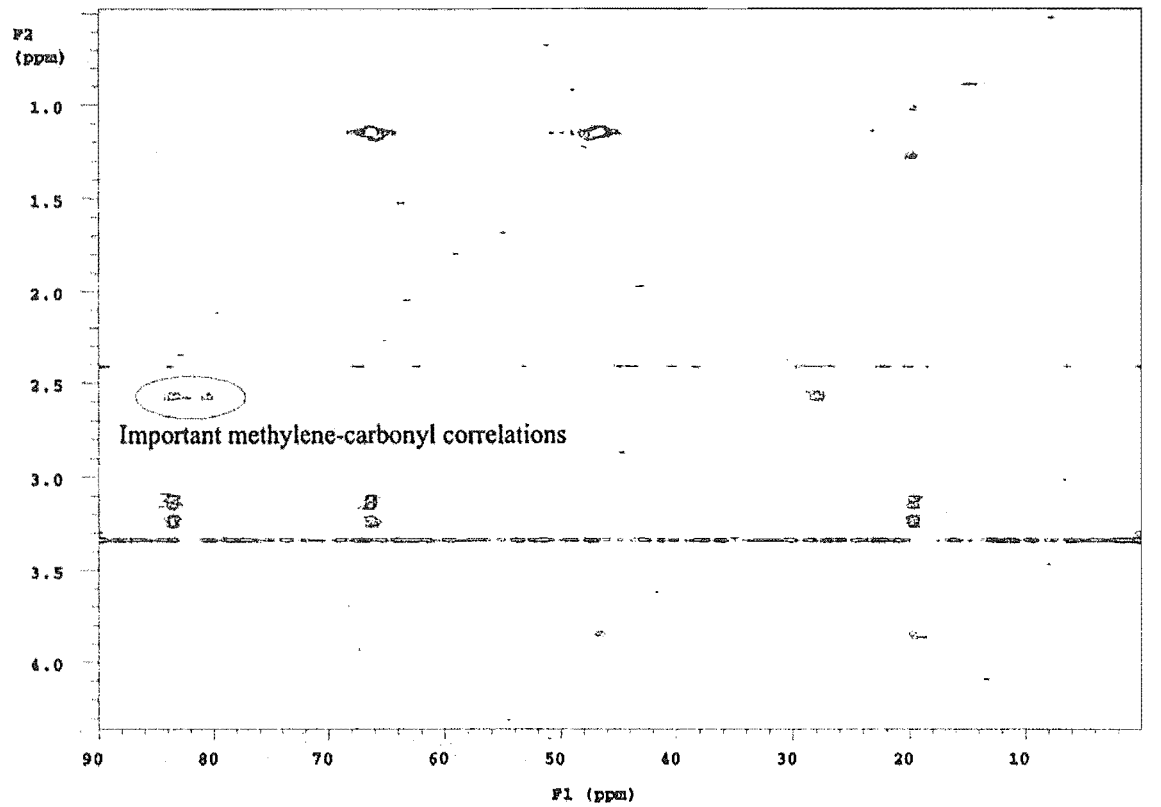
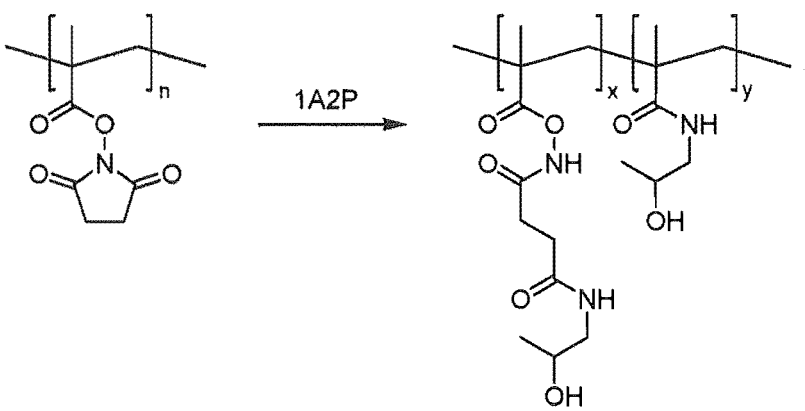


Figure 4.7: CIGAR NMR spectrum, taken in CD_3OD, of copolymer **4.3**.

Taken together, these results are consistent with pMAOS having undergone some degree of ring-opening reaction of *N*-hydroxysuccinimide (NHS) to give succinamide moieties, with the resulting non-equivalent pairs of methylene and carbonyl groups. This ring-opening is proposed to occur through attack by 1A2P at an imide carbonyl to give copolymer **4.3** (Figure 4.8), rather than the expected complete displacement of NHS through attack at the ester carbonyl.



4.3

Figure 4.8: Formation of copolymer **4.3** from the reaction of pMAOS with 1A2P.

This ring-opening of NHS-activated esters by amines has been reported previously in reactions in which either the active ester carbonyl group or the incoming nucleophile is sterically congested.[122,123] There is also strong evidence to suggest that the steric bulk around the activated ester carbonyls of pMAOS appreciably decreases the displacement of NHS by amine nucleophiles.[124] Ring-opening of NHS by primary amines has also been reported to proceed to completion at room temperature in less than 1 h,[125] and has been reported as an efficient way of synthesising *N*-(*O*-carbamoyl)-succinamides from *O*-succinimidyl carbamates.[126]

Given below is a fully assigned ^{1}H NMR spectrum of copolymer **4.3** (x = 0.62, y = 0.38; Figure 4.9). It is important to note that this spectrum has been taken in d_6-DMSO, to allow detection of the amide and hydroxamate protons, while the 2D spectra presented above were taken in d_4-methanol. This difference in NMR solvents results in small differences in chemical shifts of equivalent signals. Also, the assignment of the two sets of 1A2P signals (eg, H-12 vs H-12') is speculative and based on peak broadnesses, with peak broadness decreasing with increasing distance from the polymer backbone. Signals were initially assigned by comparison with spectra of pure pHPMA, and were confirmed by the 2D NMR experiments.

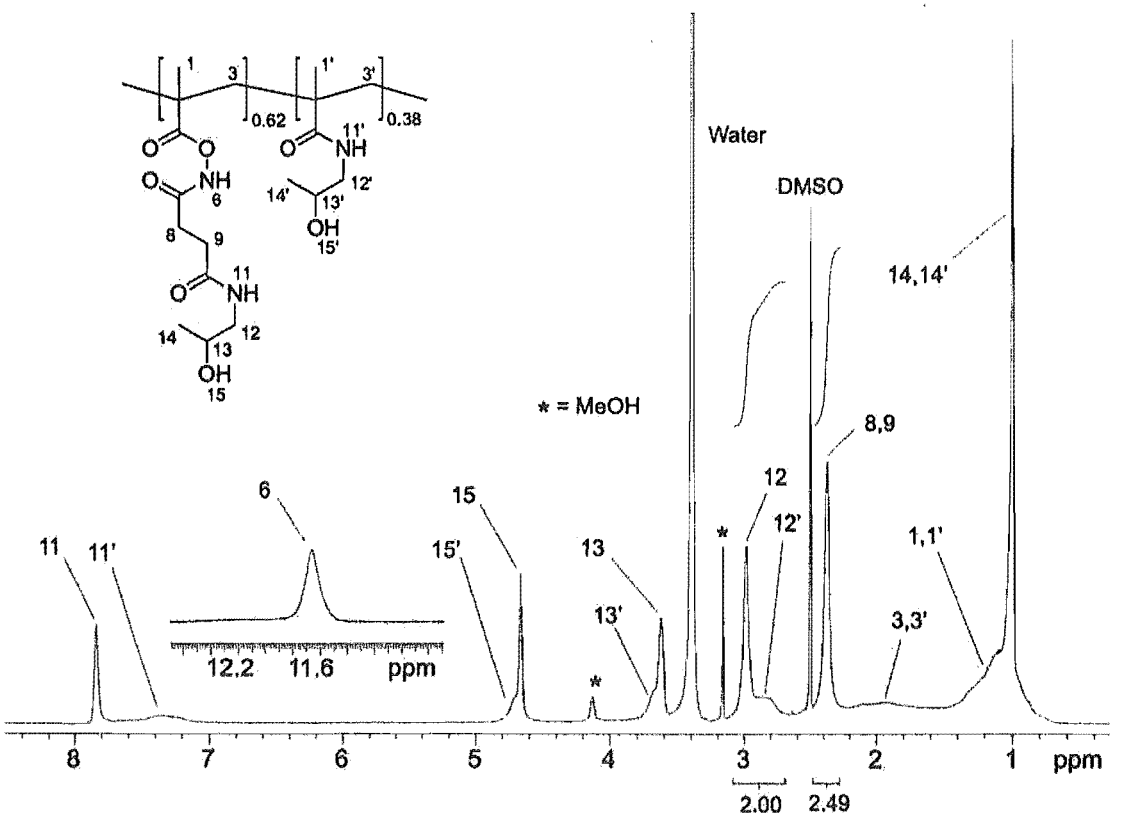


Figure 4.9: Fully assigned ^{1}H NMR spectrum of copolymer **4.3** in d_6-DMSO.

This initial work was carried out using pMAOS synthesised by ATRP, and so catalysis of this ring-opening reaction by traces of copper was considered a possibility.[127] This hypothesis was discounted, however, when RAFT-synthesised samples of pMAOS were shown to react in exactly the same fashion.

Three representative examples of the reaction of pMAOS with 1A2P are given in Table 4.3. Reaction solutions were directly purified by LH20 size-exclusion chromatography (SEC), and the polymeric material examined by ^{1}H NMR spectroscopy.

Expt.	Temp. (°C)	Time (h)	1A2P (mol eq)	Yield (%)	% Ring-opening[a]
1	40	7	1.0	84	60-65
2	40	25	1.0	85	60-65
3	50	3	1.0	91	60-65

[a] As determined using ^{1}H NMR spectroscopy - the integral of the succinamidyl methylene peak was quantified relative to the 1A2P methylene signal. Obtaining accurate integrals was often compromised by uneven baselines, and so percentages are given as multiples of 5, in accordance with this margin of error.

Table 4.3: The extent of ring-opening in the reaction of pMAOS with 1A2P.

Ring-opening, then, dominated the type of nucleophilic attack by 1A2P at the temperature that was reported to afford polymer therapeutics of type **1.1**. Lower reaction temperatures made little or no difference to the extent of ring-opening, and the copolymer product was stable in solution following its formation. Undisplaced NHS was not observed in any of the ^{1}H NMR spectra.

4.3.2.2 Hydroxamic acids isolated and characterised

It was expected that these ring-opened hydroxamate moieties would be displaced from the polymer backbone by a second attack of 1A2P (pK_a hydroxamic acids ~ 9), though at a slower rate than the displacement of NHS (pK_a ~ 8). Indeed, hydroxamate-activated esters have been used in the acylation of amines.[128,129]

Table 4.3 (above) shows that when one equivalent of nucleophile was used, the extent of ring-opening was consistently 60-65%. However, when higher stoichiometries of the amine nucleophile were used, less ring-opening was observed in the polymeric product. The reason for

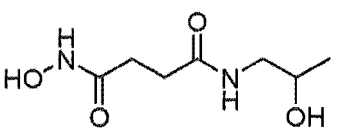
this was presumed to be due to expulsion of the hydroxamate leaving groups, formed *in situ*, by a second nucleophilic attack by 1A2P at the still-activated ester carbonyl. In support of this hypothesis, longer reaction times and higher temperatures were seen to give copolymers bearing less ring-opened material, presumably through an exacerbation of this loss of ring-opened material from the polymer backbone by a second nucleophilic attack. Table 4.4 gives the results from three representative reactions. One can infer from these results that the expulsion of the hydroxamate leaving group from the polymer backbone is indeed a slower reaction than the expulsion/ring-opening of the NHS residues.

Expt.	Temp. (°C)	Time (h)	1A2P (mol eq)	Yield (%)	% Ring-opening[a]
1	45	3.5	1.2	87	55-60
2	50	3	2.0	82	55
3	50	16	2.0	90	10

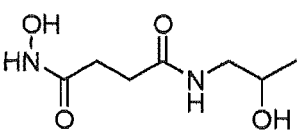
[a] As determined using ^{1}H NMR spectroscopy - the integral of the succinamidyl methylene peak was quantified relative to the 1A2P methylene signal. Obtaining accurate integrals was often compromised by uneven baselines, and so percentages are given as multiples of 5, in accordance with this margin of error.

Table 4.4: The extent of ring-opening with an excess of 1A2P.

Examination of the low-molecular weight material obtained from these reactions (following LH20 SEC) revealed a number of ring-opened succinamide derivatives, as well as the expected NHS. The two main ring-opened compounds were purified and characterized by Dr Devenish, and shown to be the *E*- and *Z*-isomeric hydroxamic acids **4.4** and **4.5** (Figure 4.10).[94]



4.4



4.5

Figure 4.10: Hydroxamic acids **4.4** and **4.5**.

Importantly, the chemical shifts for the succinimidyl methylenes were 2.18 and 2.33 for hydroxamic acid **4.4** and 2.38 (both coincident) for hydroxamic acid **4.5** in d_6-DMSO, which is

in close agreement with the value of 2.4 ppm seen with copolymer **4.3** (see Figure 4.9), lending further support to the proposed structure of this compound.

4.3.2.3 How are hydroxamic acids 4.4 and 4.5 formed?

The isolation of hydroxamic acids **4.4** and **4.5** from the reaction mixtures was taken as evidence for the proposed structure of copolymer **4.3**, however, the possibility that these compounds had been formed from free NHS in solution, following its liberation from the polymer backbone, could not be discounted. Indeed, both **4.4** and **4.5** were recovered from a reaction involving only NHS and 1A2P in methanol at 40 °C (in an experiment performed by Dr Devenish that emulated a literature report).[130]

In order to resolve this ambiguity a pure sample of copolymer **4.3**, which had no NHS groups as determined by ^{1}H NMR spectroscopy, was reacted with 1A2P. Following LH20 SEC, the isolated low molecular weight material was examined by ^{1}H NMR spectroscopy and seen to contain the hydroxamic acids **4.4** and **4.5** (it should be noted that these compounds had partly reacted further to give related compounds that were not characterized). As there was no source of NHS in this experiment, **4.4** and **4.5** must have initially been conjugated to the polymer backbone, as in structure **4.3**. This was taken as final proof of the structure of **4.3**.

Having proved structure **4.3**, attempts must be made to explain why the ring-opening reaction was so pronounced in this system, yet not in others to the same extent. The reason is presumably related to steric hindrance at the ester carbonyl centre. To test this idea, a solution of *N*-hydroxysuccinimidyl pivalate (**2.23**; see Section 2.3.7) was heated with two equivalents of 1A2P in DMSO at 55 °C for three hours. Analysis of the crude reaction mixture by MS (positive mode) revealed the expected amine product (which dominated the spectrum), while no ring-opened hydroxamate ester material was observed. The products were isolated using a C_{18} cartridge, and the material shown, through the use of ^{1}H NMR spectroscopy, to be exclusively the amide product.

This result shows that the quaternary α-centre alone does not account for the presumed steric hindrance of the pMAOS carbonyl centers. It is proposed, then, that the polymer conformation in solution exacerbates this steric hindrance, leading to extensive ring-opening of the succinimide residues.

4.3.2.4 Can copolymer 4.3 replace pMAOS in the synthesis of polymer therapeutics?

The observation, described in Sections 4.3.2.2 and 4.3.2.3, that copolymer **4.3** still represented an activated polymer backbone led to the idea that **4.3** could be used in place of pMAOS in the synthesis of polymer-drug constructs of type **1.1** (via the reaction scheme shown in Figure 4.5). Therefore, pMAOS was reacted with 1A2P to give copolymer **4.3** *in situ* (assumed to be 60-65% ring-opened), conjugate **3.26** (0.1 eq) was added, the reaction stirred at 70 °C for 22 h, then 1A2P (2 eq) added and the reaction stirred for an additional 17 h at 70 °C. The reaction mixture was purified by LH20, and the high molar mass and low molar mass material (both yellow in colour) collected for characterisation.

The polymeric material was examined by ^{1}H NMR spectroscopy. It was difficult to obtain reliable integrals, due to an uneven baseline, but a comparison of the peak integrals for the 9-NH proton of the variolin component of conjugate **3.26** with the polymeric 1A2P methylene signal suggested that the polymer was carrying 5 mol% variolin (this result was later refined to 2.5% based on results, described in Section 4.3.2.5, which revealed further structural characteristics of the polymer – namely, the presence of glutarimide moieties). It was decided that this result should be checked by UV quantification.

UV analysis of a standard solution of conjugate **3.26** in DMSO gave an extinction coefficient at 410 nm ($\varepsilon_{(410\ nm)}$) of 20 000 $Lmol^{-1}cm^{-1}$. The UV absorbance at 410 nm of a 250 μg/mL solution of the polymeric material, performed in triplicate, gave an average $A_{(410\ nm)} = 1.02$. From this, a concentration for polymer-bound conjugate **3.26** of 51 μM was inferred, which gave a molar loading of 3.3% for the conjugate (this result was later refined to 2.5%, based on results described in Section 4.3.2.5, which revealed further structural characteristics, and therefore the correct molar mass, of the polymer). This was, in hindsight (that is, after the values had been adjusted due to the aforementioned refined copolymer molecular formula that included glutarimide units – see Section 4.3.2.5), in good agreement with the NMR result. At the time, however, an ambiguity remained (5 mol% vs 3.3 mol%), and so it was decided to investigate the low molar mass material in an attempt to quantify the amount of conjugate **3.26** in this fraction.

Analysis by ^{1}H NMR spectroscopy, not surprisingly, showed the low Mr fraction to be comprised mainly of ring-opened succinamide material, a small amount of NHS, and a small amount of conjugate-like material. The mixture was complex, however, so no reliable information regarding the amount or identity of the conjugate-like material could be gleaned.

The UV profile of this low molar mass material was typical of variolin-containing material, however due to the complexity of the mixture UV quantification was considered unreliable. Investigations using HPLC were therefore pursued.

Injections of solutions of conjugate **3.26** (3.4×10^{-9} mol) onto the HPLC, run with the standard gradient elution protocols and analysed at 248 nm, gave average peak areas of 1.03×10^{12} AU.min.mol^{-1}. Dissolution of the low molar mass material from the attempted polymer therapeutic synthesis in DMSO (3 mL), followed by a 1:100 dilution and injection of 100 μL of the resulting solution onto the HPLC (in triplicate) gave a series of five peaks, all with conjugate-like chromophores, eluting between 10 and 12.5 minutes. One of these peaks, eluting at 10.2 minutes and comprising less than 5% of the total peak area of the region, was confirmed by sample spiking to be conjugate **3.26**. The combined peak areas of all the conjugate-like peaks was 4400 AU.min. Assuming that the extinction coefficients for these compounds at 248 nm were the same as for conjugate **3.26**,* then 85% of the conjugate starting material could be accounted for by these unidentified compounds in the low Mr faction. This was in excess of the expected value, though only by 10% (given a molar loading of 2.5% on the polymeric material). This discrepancy may be explained by a change in extinction coefficients for the conjugate side-products, by other non-conjugate-derived impurities also eluting from the HPLC column at 10-12 minutes (a distinct possibility, given the complexity of the region), or simply by the error margin of the investigation. The result nonetheless corroborates the conclusion that only a fraction of the conjugate **3.26** starting material, probably ~2.5%, ended up on the polymer backbone.

None of the four conjugate-like side products identified by HPLC were characterised (LCMS analysis gave insufficient information for even speculation). Their appearance was attributed to the relatively high reaction temperature and long reaction times needed. Subsequent results (reported in Section 4.3.2.5) led to this line of work being abandoned, rendering the characterisation of these compounds redundant.

Although TEA has been reported to decrease the rate of acylation of amines by hydroxamate esters,[129] the attempted polymer therapeutic synthesis from copolymer **4.3** was repeated with the

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* A reasonable assumption, based on the observation that the UV absorbancy of the reaction mixture was seen to change only slightly during the course of the reaction, as is discussed below.

inclusion of TEA, in an attempt to minimise the unwanted side reactions. Unfortunately, the glutarimide-forming side reaction (described in Section 4.3.2.5) was seen to occur at prohibitively high levels. These experiments did, however, show that a reaction time of seven hours at 45 °C was sufficient to give complete reaction of conjugate **3.26**, with ~70% of the conjugate ending up on the polymer backbone. Most of the variolin material remained polymer-bound during the subsequent reaction with 1A2P.

A further important result was that the UV absorbance at 410 nm of a reaction solution did not change significantly during the course of a reaction, strongly suggesting that the extinction coefficient of conjugate **3.26** was not significantly altered by conjugation onto a polymer backbone up to a concentration of at least 7 mol% substitution. This validated the method for quantification of variolin material on a polymer backbone by UV spectrophotometry.

As was alluded to above, in an attempt to reduce conjugate **3.26** consumption through unwanted side reactions, subsequent polymer therapeutic synthetic attempts were carried out employing lower reaction temperatures. Examination of the polymeric products by ^{1}H NMR spectroscopy, however, consistently gave spectra with anomalous integrals; specifically the polymeric 1A2P oxymethine signal integrated too high when compared with the 1A2P methylene signal, and both were less than expected when compared to the backbone signals. Reexamination of the spectra obtained from the higher temperature reactions showed the same discrepancies. Furthermore, some of the polymeric products were eventually found to be water insoluble.

These observations were investigated more closely, again employing a model system using only 1A2P as nucleophile. What was discovered is described in Section 4.3.2.5, and would lead to the abandonment of the synthesis of polymer therapeutics from copolymer **4.3**.

Before moving on and discussing the nature of this second side reaction, it should also be noted here that none of the polymeric constructs synthesised possessed demonstrable bioactivity (that is, an IC_{50} value against the P388 cell line of <125 μg/mL).

4.3.2.5 Hydroxamate expulsion to give glutarimide moieties

The formation of water-insoluble polymers from the reaction of pMAOS with primary amines was reported by Ringsdorf in his 1975 paper.[17] In this paper, he briefly mentions that the reaction of pMAOS with ethanolamine gave the expected water-soluble polymer when the reaction was carried out below 40 °C, but water-insoluble polymers resulted when the reaction

was carried out at temperatures greater than 40 °C. The insoluble polymers were presumed to be cross-linked polymers, formed by the reaction of alcohol groups of polymer-bound ethanolamide moieties with unreacted NHS ester groups. No further experimental or characterisation details were given. Ethanolamine is a close structural analogue of 1A2P, and so this report was seen to corroborate the findings described above. However, it was considered desirable to investigate the water-insoluble polymers more closely in order to confirm their structure.

When copolymer **4.3** was heated in DMSO with 1A2P the polymeric product was insoluble in water, and displayed the integral anomalies described above when examined by ^{1}H NMR spectroscopy (see Figure 4.14). Importantly, however, the NMR spectra contained 1A2P hydroxyl signals that were larger than would have been expected had significant amounts of ester cross-links been formed. This result strongly challenged the explanation previously offered for the formation of these insoluble polymers, and so 2D NMR experiments were again employed to elucidate the structure of this polymer.

An HSQC-DEPT experiment (Figure 4.11) revealed a very broad set of methylene signals that had previously escaped attention spread across ~0.5 ppm (~3.4-3.9 ppm) and partially obscured by the oxymethine signal (3.8-4.0 ppm) (see Figure 4.14). The ^{13}C shift of the associated carbons suggested that these signals were 1A2P methylene groups. Further support for this assignment came from a COSY experiment (Figure 4.12), which showed a correlation from this broad region to the oxymethine signal.

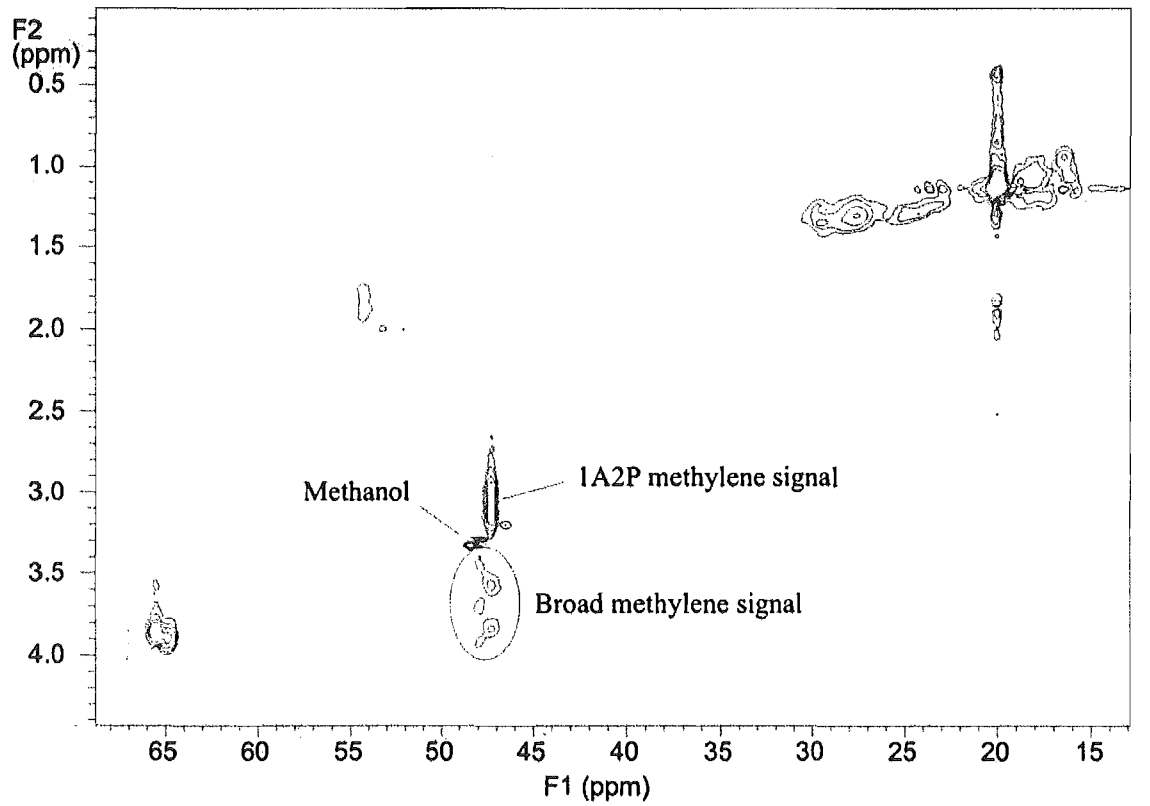


Figure 4.11: HSQC-DEPT spectrum of copolymer **4.6**, taken in CD_3OD.

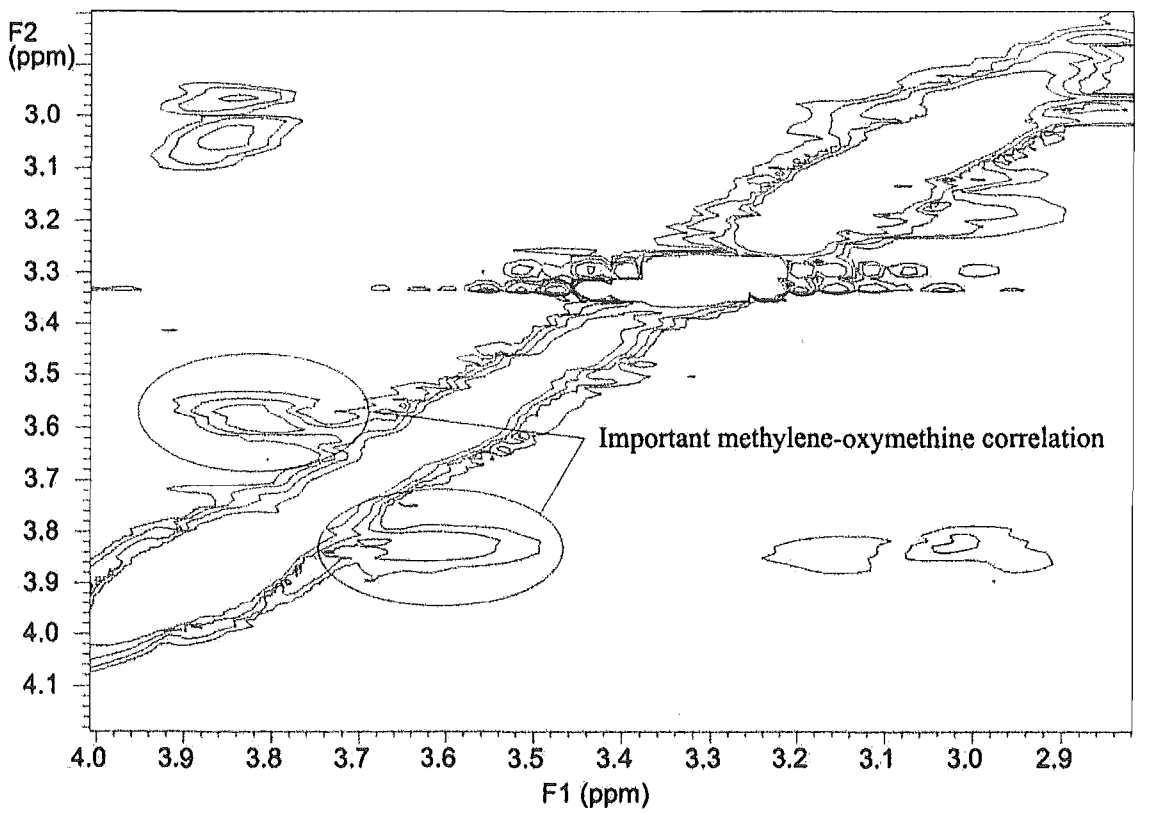
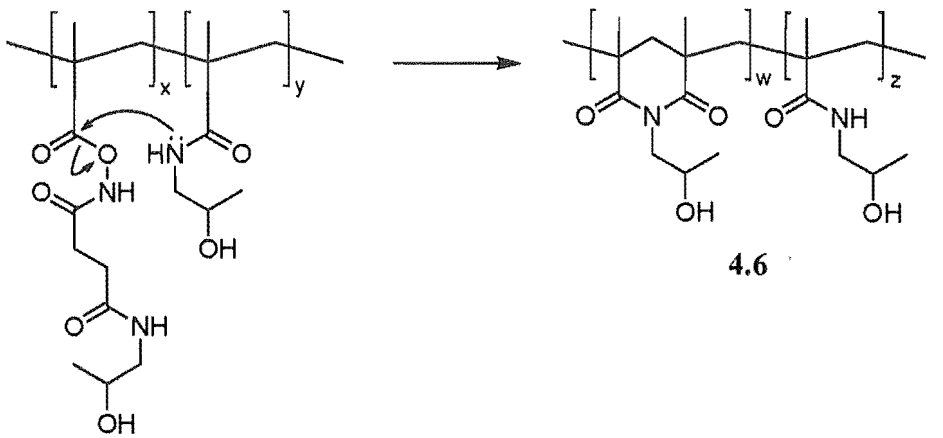


Figure 4.12: COSY spectrum of copolymer **4.6**, taken in CD_3OD.

Taken together, these data indicated that a copolymer containing 1A2P residues in both amide and imide functionalities, copolymer **4.6**, had been formed (Figure 4.13). The formation of **4.6** is proposed to occur through ring-closing attack of amides on (presumably) neighboring hydroxamate-activated esters to form *N*-substituted glutarimides.



4.6

Figure 4.13: Formation of copolymer **4.6** from copolymer **4.3**.

The ^{1}H NMR data were consistent with this proposed structure, with the oxymethine protons of the glutarimide and HPMA moieties being coincident and partially overlapping the very broad signal from the 1A2P methylene protons of the *N*-substituted glutarimide (see Figure 4.14).

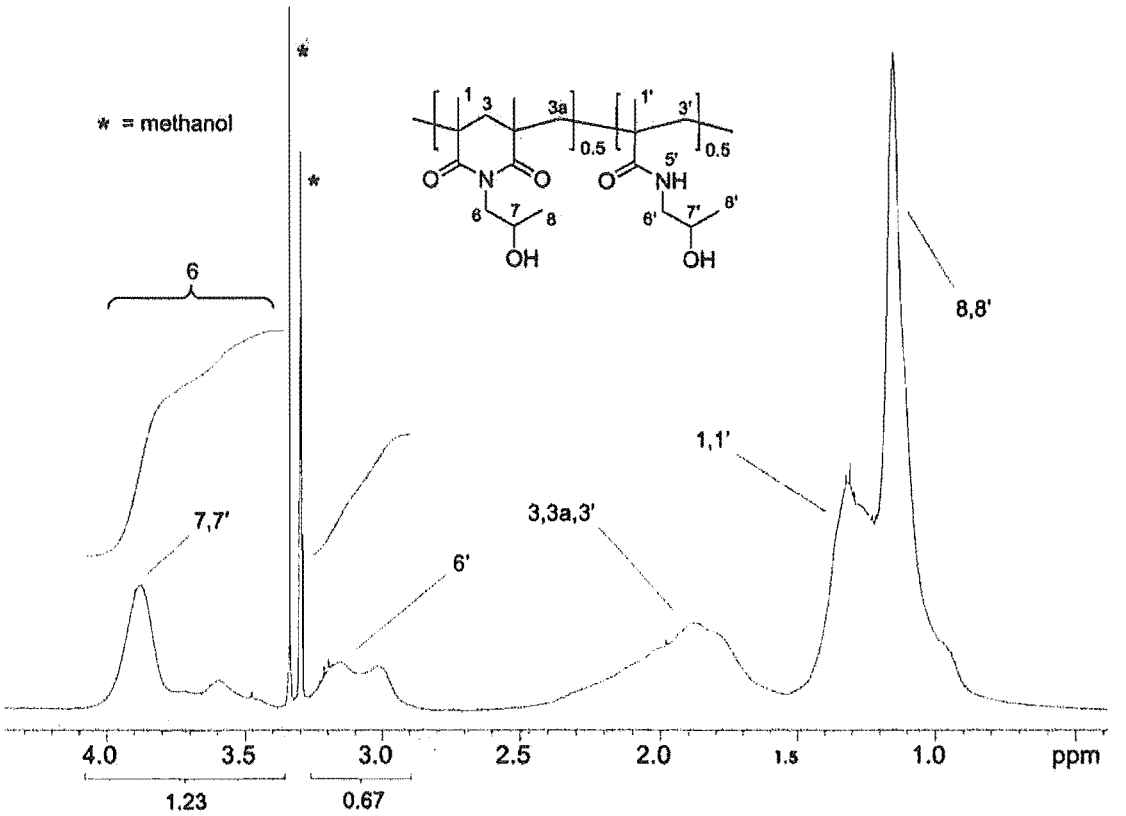


Figure 4.14: Fully assigned ^{1}H NMR spectrum of copolymer **4.6** in d_4-methanol.

To further prove this structure, a pure sample of ring-opened copolymer **4.3** in d_6-DMSO was heated at 70 °C in the absence of 1A2P and the reaction followed by ^{1}H NMR spectroscopy. The liberation of hydroxamic acids **4.4** and **4.5** was clearly observed during the course of the experiment, and after completion copolymer **4.6** was isolated from the reaction mixture.

It was found that this ring-closing reaction dominated any attempts to convert copolymer **4.3** to pHPMA. When **4.3** (~65% ring-opened) was heated in 50% 1A2P/DMSO at 45 °C for 24 hours, copolymer **4.6** was isolated with an HPMA:glutarimide ratio of ~1:1, indicating that ~50% of the hydroxamate displacement from **4.3** had occurred through intramolecular attack.

Glutarimide formation during the aminolysis of pMAOS has been previously reported; specifically, 1-5% glutarimide formation was reported to occur at ambient temperature, with no observable formation at temperatures of 50-60 °C.[61] It is likely that the researchers were in fact observing the presence of ring-opened material in their ^{1}H NMR spectra, though no NMR characterisation data were given, and so further comment can not be made here regarding this report.

4.3.2.6 Implications for the failed polymer therapeutic syntheses from pMAOS

It was now clear why the efforts toward a synthesis of a polymer therapeutic carrying conjugate **3.26** using pMAOS as the polymeric precursor (described in Section 4.3.1.1) were unsuccessful. The majority of the yellow variolin-containing material had ended up in the low Mr fraction following LH20 SEC, with less than 2 mol% remaining on the polymer. It is now known that the two reactions shown in Figure 4.15 would have represented a significant loss of variolin material from the polymer.

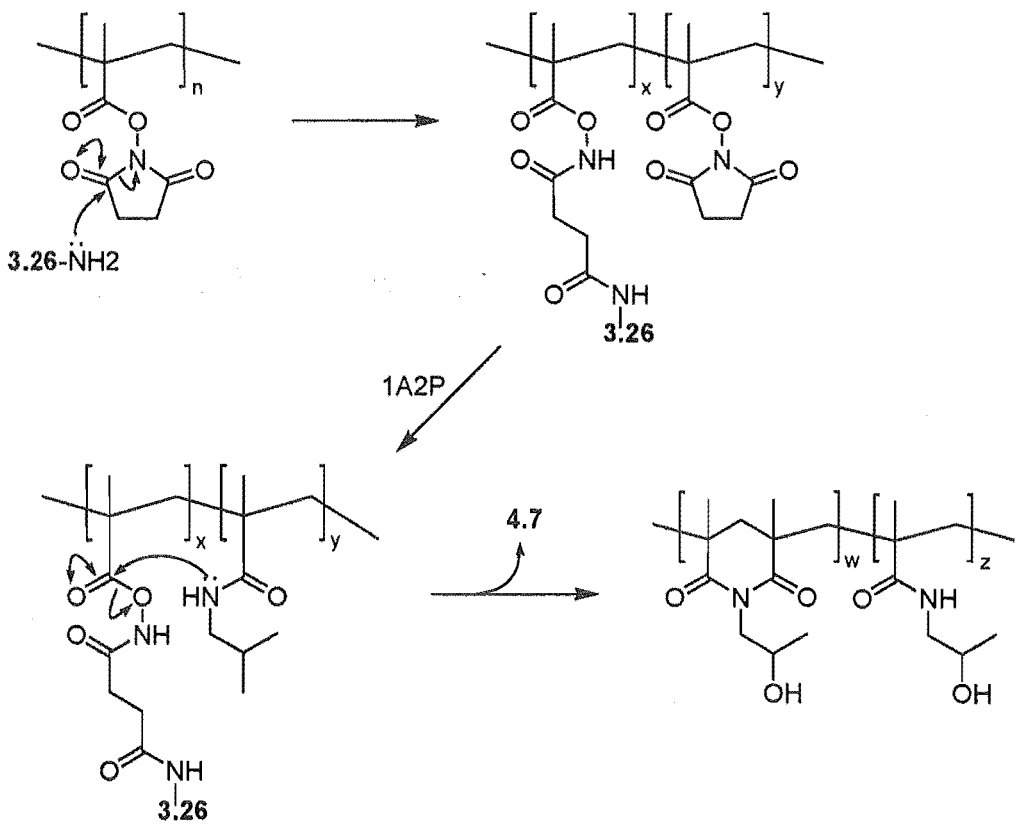
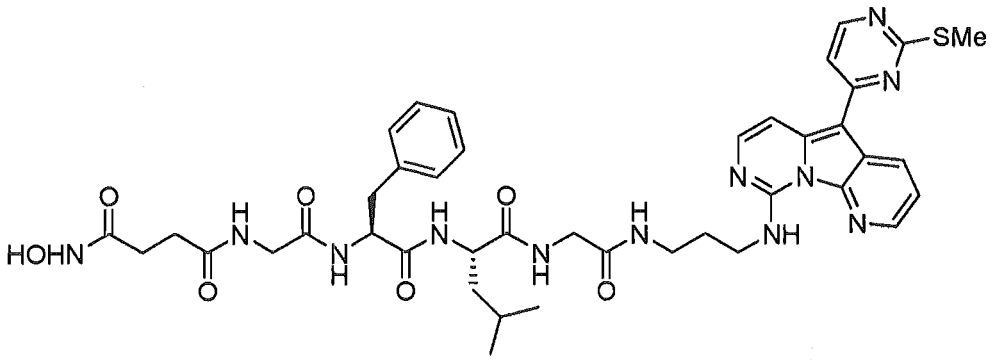


Figure 4.15: Competing side-reactions during the attempted synthesis, using pMAOS, of a polymer therapeutic incorporating conjugate **3.26**.

The material, **4.7**, that was expelled from the polymer backbone in the third and final step of the above reaction scheme is shown below in Figure 4.16.



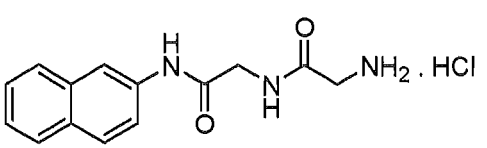
4.7

Figure 4.16: Leaving group **4.7**.

Attempts at identifying leaving group **4.7** in the low Mr fraction of the attempted polymer therapeutic syntheses were met with only partial success – analysis by LCMS showed a compound of the expected molar mass, though apparently at significantly lower concentrations than would have been expected. The most likely reason for this is that **4.7** was presumed to have reacted further to give other unidentified compounds (an idea taken from the observation that hydroxamic acids **4.4** and **4.5** were isolated from complex reaction mixtures comprised of other similar compounds).[130]

4.3.2.7 Replication of the Godwin/Brocchini experiment

In a final attempt to utilize pMAOS, it was decided that the reaction conditions previously reported to provide the HPMA copolymer **1.3** (see Section 1.2.3.2) would be replicated exactly.[60] Firstly, *N*-glycyl-glycyl-β-naphthylamide hydrochloride, **4.8** (Figure 4.17), was synthesised from β-naphthylamine via conventional solution-phase peptide synthetic protocols in 44% yield over four steps.



4.8

Figure 4.17: The drug substitute, **4.8**, used in the reported model synthesis of a polymer therapeutic.

This material was then used in the reaction shown in Figure 4.18.

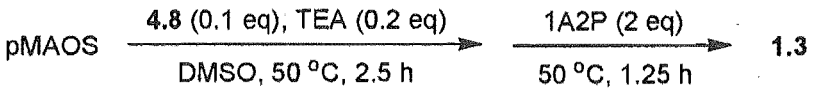
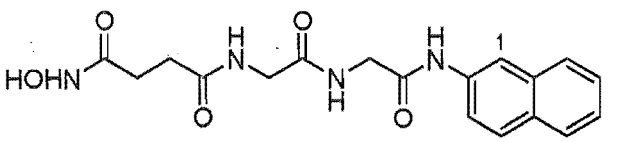


Figure 4.18: An exact replication of the reported protocols for the synthesis of the HPMA copolymer **1.3**, bearing 10 mol% **4. 8**.

The crude reaction mixture was analysed by HPLC, using the standard gradient elution protocols, and shown to contain small amounts of two low Mr chromophore-containing compounds (eluting as sharp peaks), with the majority of the chromophore being polymer-bound and eluting as a broad peak from 9-15 minutes. Of the two low Mr compounds, one eluted at 8.5 minutes and was shown by sample spiking to be unreacted conjugate, and the other eluted at 10.1 minutes, and was presumed to be leaving group **4. 9** (Figure 4.19).



4.9

Figure 4.19: Leaving group **4.9**.

Purification of the reaction mixture by LH20 SEC gave a polymeric product and a low Mr fraction. Examination of the polymeric product by ^{1}H NMR spectroscopy showed that the polymer contained 60-65 mol% ring-opened material. Comparing the integrals for the H-1 of the naphthylamide moiety with the polymer backbone and 1A2P methyl signals suggested that the polymer was carrying 9-10 mol% conjugate **4.8**. This was in agreement with the HPLC result obtained with the crude reaction mixture. Examination of this material by FTIR showed poorly-defined absorbances throughout the spectra, with a complete absence of the NHS-ester carbonyl peak at 1735 cm^{-1} (the latter result was consistent with the disappearance of the active ester peak reported in the literature).[60,62]

The subsequent reaction of this polymeric material in the presence of 1A2P at 50 °C for 2 d gave a copolymer with an HPMA:glutarimide ratio of ~2:1. Integrals also suggested that the polymer was carrying 5-6 mol% conjugate **4.8**. The isolated copolymer was again examined by FTIR spectroscopy, which again showed only poorly-defined absorbances throughout the spectra, with no strongly distinguishing signals from that of the copolymer isolated after the first step of the

reaction. Formation of copolymers of type **4.3** and **4.6**, therefore, cannot be readily observed by IR spectroscopy.

4.3.3 Reaction of pMAOS with benzylamine

A 2004 paper, coauthored by Monge and Haddleton, describes the synthesis of pMAOS by copper-mediated ATRP.[62] Polymers with $\bar{M}_n \sim 4000$ gmol^{-1} and PD < 2.0 were synthesised, and then used in the synthesis of poly(*N*-benzyl methacrylamide). The displacement reaction was carried out at 50 °C for five hours – the reaction was followed by IR spectroscopy and was stopped upon the complete disappearance of the NHS-activated ester carbonyl peak at 1736 cm^{-1}. The reaction solution was concentrated *in vacuo* and the polymer reportedly purified by precipitation from DMSO with methanol. A ^{1}H NMR spectrum given in the paper of the polymeric product at first glance appears to be pure poly(benzyl methacrylamide), free of any ring-opened succinimidyl moieties.

It is obvious that this polymer contains no significant amounts of ring-opened material. However, close inspection reveals the likely presence of some glutarimide material. While integral ratios are not given, a manual measurement of the peak areas (ie, cutting out and weighing each peak) suggested that the polymer was comprised of both amide and glutarimide moieties. The hypothesised *N*-substituted glutarimide benzyl methylene signal is a slightly raised region ~0.4 ppm upfield of the amide benzyl methylene signal. Given the small size and broadened nature of this signal reliable integrals could not be obtained, however a rough estimate would suggest that the glutarimide:benzyl methacrylamide ratio was ~1:10.

In order to confirm this assertion, it was decided that the reaction would be replicated. Thus, a solution of pMAOS and benzylamine was stirred at 50 °C for five hours. The reaction was performed in triplicate. One reaction was purified immediately by LH20 SEC, and the other two were left under reduced pressure (0.03 mm Hg) at room temperature for seven days to remove the liquid reagents. This was in accordance with the Monge and Haddleton protocols, though neither times nor temperatures were given in their paper for this concentration process. Of the latter two, one was purified by LH20 chromatography, while the second was dissolved in a minimal amount of DMSO then diluted 100-fold with methanol. No precipitation occurred in this final experiment; furthermore, methanol had successfully been used as the eluant during the LH20-based purification of the polymeric material from the first two reactions. It is not known why this discrepancy between what was reported and what was observed exists.

The polymeric material from the first reaction (ie, that which was immediately purified) was analysed by ^{1}H NMR spectroscopy and shown to be a copolymer carrying both ring-opened succinimide and glutarimide material (Figure 4.20). The assignment of the glutarimide benzyl methylene signal at 4.7 ppm was supported by an HSQC-DEPT NMR experiment. This signal is in the same position as the presumed glutarimide methylene signal seen in the ^{1}H NMR spectrum given in the Monge and Haddleton paper.

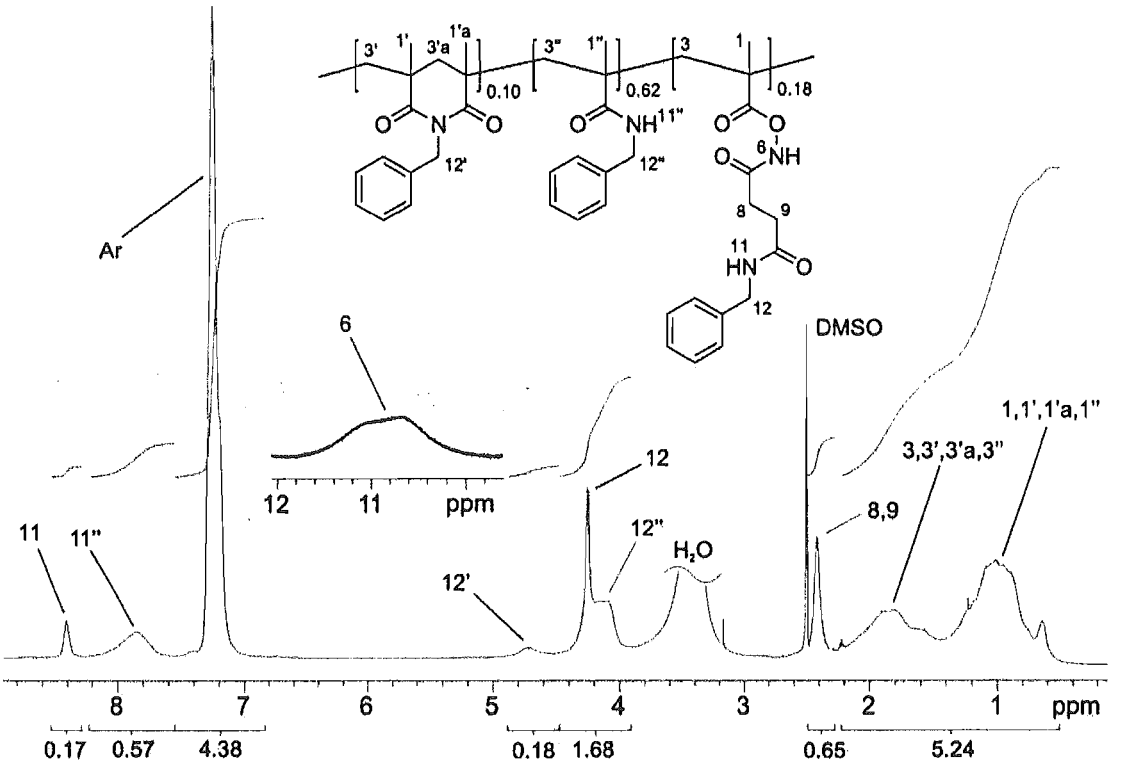


Figure 4.20: ^{1}H NMR spectrum, taken in d_6-DMSO, of the polymeric product from the reaction of pMAOS with benzylamine.

The amount of ring-opened material observed was less than expected. This may reflect different conformations in solution (and therefore different degrees of steric hindrance) of the polymer intermediates between the 1A2P and benzylamine substitution reactions. Conversely, the amount of glutarimide formation is higher than expected. No explanation can be given for this, although Section 4.4.1.2 (below) does show that the ring-closing reaction can be relatively facile in some systems.

Analysis of the polymer isolated from the second reaction (that is, after seven days under reduced pressure) showed it to be fairly similar to that shown above, with the only difference being that it carried less ring-opened material and more benzyl methacrylamide. The ratio of glutarimide : benzyl methacrylamide : ring-opened units in this polymer was about 1 : 7 : 1.

Therefore, during the seven days under reduced pressure slightly less than half of the hydroxamate moieties were displaced by a subsequent attack of benzylamine. Very little (if any) glutarimide formation occurred during this time, which is consistent with earlier observations suggesting that this reaction is less pronounced at lower temperatures.

While no ring-opened material was seen in the polymer made by Monge and Haddleton, a likely explanation for this is that hydroxamate material was indeed formed, but subsequently expelled upon attack by benzylamine during the sample concentration phase. (The temperature at which this concentrating step was carried out is not given.) The amount of ring-closing that can be seen in their polymer sample is comparable to that found in this work.

Another possibility is that that the pMAOS samples that were made and used in their work were from 3.6 to 4.5 kDa. This is around 7 times smaller than the RAFT-synthesised polymer used in the work described here ($\bar{M}_n$ ~ 30 kDa). It is possible that the smaller molecules may adopt a different conformation in solution, so that steric hindrance around the ester carbonyls is minimised. This idea was not investigated further, however.

4.4 Other Activated Polymers

With conjugate **3.26** still in hand, it was decided that other activated methacrylic homopolmers would be investigated, in the place of pMAOS, as possible precursors to polymeric constructs of type **1.1** in the type of reaction shown in Figure 4.5.

4.4.1 Poly(*p*-nitrophenyl methacrylate) (pNPMA)

Given than *p*-nitrophenol-activation is currently employed in the conventional synthetic route to polymer therapeutics (see Section 1.1.3.1), it was decided that a pNPMA homopolymer (Figure 4.21) would be investigated as a possible substitute for pMAOS in the protocols being employed in this work.

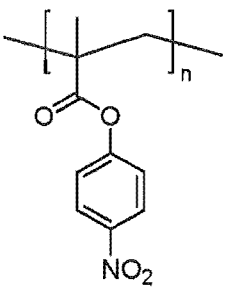


Figure 4.21: pNPMA

4.4.1.1 Synthesis of pNPMA

pNPMA homopolymer was synthesised by Adash from *p*-nitrophenyl methacrylate via conventional free radical polymerisation. GPC analysis was not performed on this sample, which was synthesised with an M_{target} of 20 kDa and was presumed to be of high PD.

4.4.1.2 Reaction of pNPMA with 1A2P

The reactivity of pNPMA was investigated using 1A2P as the nucleophile. A solution of the polymer (97 μM), 1A2P (2 eq) and TEA (1.5 eq) in DMSO was stirred at 45 °C for 48 hours, and aliquots were periodically removed and analysed by HPLC using the standard gradient elution protocols at a wavelength of 315 nm. *p*-Nitrophenol eluted as a sharp, well-defined peak at 11.6 minutes (this was confirmed by sample spiking). Peak areas were measured and compared to those obtained with a standard solution of *p*-nitrophenol (6.5×10^{11} AU.min.mol^{-1}) in order to quantify the amount of leaving group displacement. The results are given below in Figure 4.22.

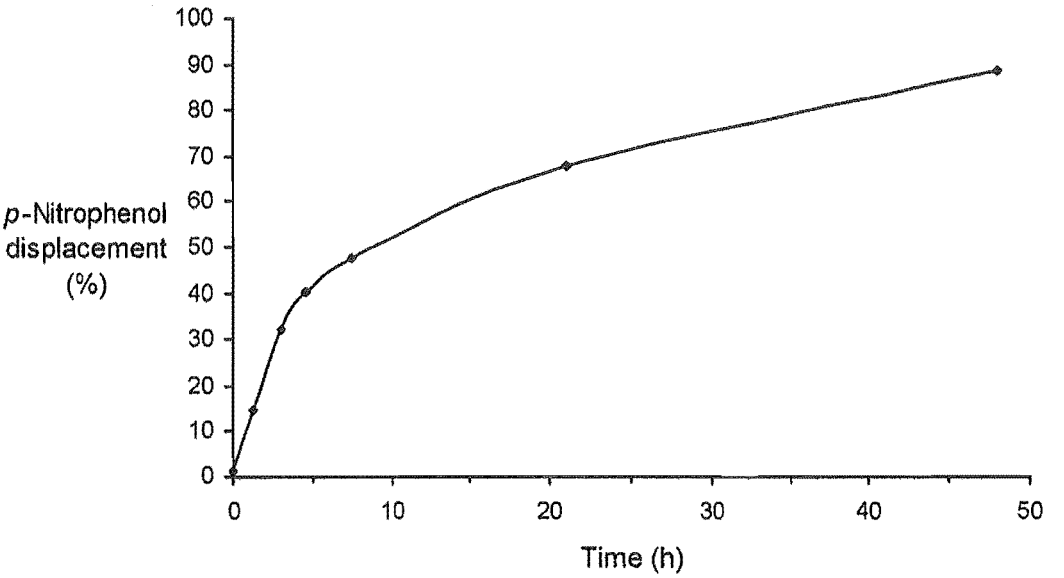


Figure 4.22: Liberation of *p*-nitrophenol from pNPMA upon reaction with 1A2P.

After 48 h 89% of the *p*-nitrophenol had been displaced from the polymer backbone, as determined by HPLC. At this time the polymeric material was purified by LH20 SEC and examined by ^{1}H NMR spectroscopy. The NMR spectrum clearly showed that a large amount of glutarimide formation had taken place, and also confirmed that ~10 mol% of the *p*-nitrophenol residues remained polymer-bound. Figure 4.23 gives the structure of the copolymer product, along with the ratios of the three different structural units.

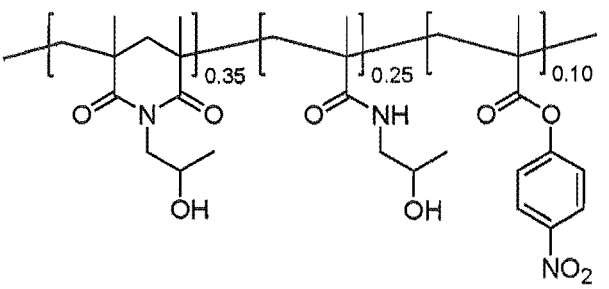


Figure 4.23: The polymeric product from the reaction of pNPMA with 1A2P.

The large amount of glutarimide formation further confirmed the proposed structure of the glutarimide moiety, with 2D NMR experiments run on this polymer again giving the key correlations. This increase in glutarimide formation in pNPMA compared with pMAOS may be due to either the increased electrophilicity of the *p*-nitrophenyl-activated ester compared with the

NHS-activated ester, a possible different polymer conformation in solution, or a combination of both.

In light of the large amount of observed glutarimide formation, this line of investigation was abandoned.

4.4.2 Poly(methacryloyl chloride) (pMAC)

A 1998 paper reported that poly-methacryloyl chloride (pMAC) gave superior results to pMAOS in the synthesis of polyamides.[131] Adash had begun work towards the synthesis of samples of pMAC of low PD (work that is still being undertaken at the time of writing), and so this polymer was investigated as a possible precursor in the synthesis of polymer-drug constructs of type **1.1**.

4.4.2.1 Synthesis of pMAC

The synthesis of pMAC by conventional polymerization protocols has been reported; AIBN was used as the initiator, and temperatures of 60-65 °C for 2-3 days gave yields of 89-95%.[131,132] Several attempts were made at synthesising pMAC by these protocols, with generally poor results. Eventually, a synthesis carried out in toluene at 85 °C for 21 hours gave a polymeric product in 28% yield (based on weight recovery, and later refined to 29% based on a presumed acid chloride/free acid copolymer structure – see below). Longer reaction times did not improve on this yield. Examination of this material by ^{1}H NMR spectroscopy showed only one set of signals, which were in the correct position for the pMAC backbone signals. These signals were very 'spikey', however, suggesting that low Mr material had been formed, most probably well short of the M_{target} of 5.5 kDa, or 53 monomer units. The spikey appearance of the backbone signals may have also reflected some degree of hydrolysis of the acid chloride moieties, possibly in the NMR solvent ($CDCl_3$). It was therefore decided that a subsample of this polymer would be converted to the methyl ester analogue poly-methyl methacrylate (pMMA) in order to quantify the amount of reactive acid chloride groups remaining on the polymer chain.

Thus, a dry solution of the freshly-made pMAC in a mixture of pyridine, methanol and THF was heated to reflux under argon for three days. The polymeric product was purified by precipitation from tetrahydrofuran with methanol in a disappointing 24% yield. Examination of this material by ^{1}H NMR spectroscopy again gave 'spikey' backbone signals, indicative of a copolymer

and/or low Mr material, with a methyl ester signal integrating to around two protons (relative to the backbone signals). This suggested that ~30% hydrolysis had occurred at some point, either during the synthesis of pMAC or the subsequent synthesis of pMMA (the monomer starting material, methacryloyl chloride, was shown by ^{1}H NMR spectroscopy to be pure, ie, free of methacrylic acid). Hydrolysis during polymer synthesis became the most likely explanation when this value of ~30% was confirmed in a separate substitution reaction involving the much stronger nucleophile 1A2P (see below, Section 4.4.2.2).

4.4.2.2 Reaction of pMAC with 1A2P

Despite the suspected low quality of the pMAC sample, it was decided that reaction of this polymer with 1A2P would be investigated. pMAC is more electrophilic than pNPMA and pMAOS (and even more so than the ring opened copolymer **4.3**), and so it was anticipated that this polymer, even if it was ~30% hydrolysed, would show a significant degree of glutarimide formation.

A sample of pMAC was reacted with five equivalents of 1A2P under dry conditions at 70 °C for 16 hours. The polymer that was isolated was examined by ^{1}H NMR spectroscopy and shown to be comprised of methacrylic acid, HPMA and glutarimide units in a ratio of 3:5:1 (Figure 4.24). This interpretation was based solely on the peak integrals; the glutarimide 1A2P methylene signal is not obvious, but this is considered reasonable given the large area over which it is spread.

Given the presumed ~30% methacrylic component of this polymeric product, and given that this value is the same as that seen in the pMMA synthetic reaction from the same starting material, it can be inferred that the pMAC starting material was most probably ~30% hydrolysed. It is not known why this hydrolysis occurred, as all attempts were made, using well-established anhydrous protocols, to rigorously exclude water from the polymerisation reaction.

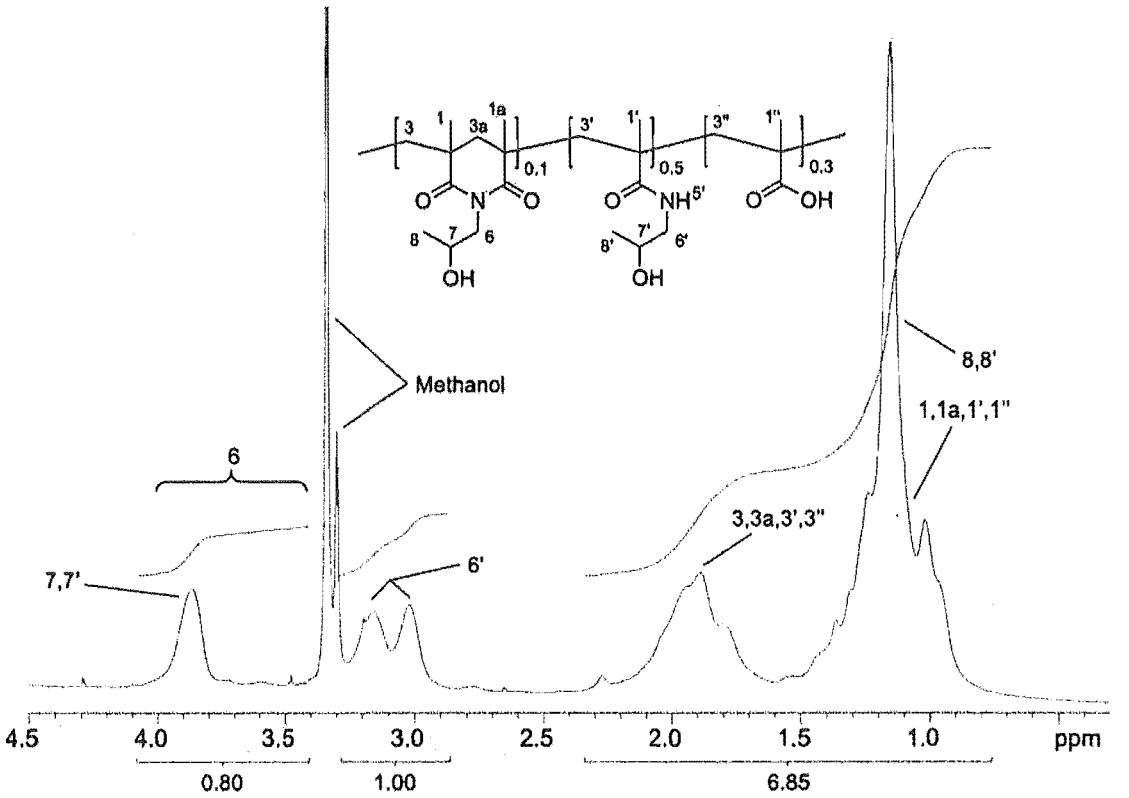


Figure 4.24: ^{1}H NMR spectrum of the polymeric product from the reaction of pMAC (presumably 30% hydrolysed) with 1A2P.

The amount of glutarimide formation would indicate that around 5-10% of the available acid chloride moieties were attacked by a neighbouring HPMA amide in the glutarimide-forming reaction. This level of glutarimide formation was less than what was expected, given the amount of glutarimide formation that was seen with the pMAOS and pNPMA. One possible explanation is that the polymer starting material was sufficiently hydrolysed so that there were few HPMA residues adjacent to methacryloyl chloride residues as the substitution proceeded, however it is considered unlikely that this alone could account for the relatively low amount of observed glutarimide formation. Another more likely possibility is that the more sterically hindered acid chloride carbonyl favours intermolecular attack by the smaller 1A2P rather than the intramolecular reaction with the larger amide group. A third consideration is that polymer conformation in solution may significantly affect the relative rates of the two types of nucleophilic attack. Furthermore, the presumed lower molar mass of the pMAC molecules used in this investigation may have affected this conformation.

4.5 Conclusions and Future Work

The use of polymethacryloyl active esters as precursors to polymethacrylamides is precluded by formation of unwanted side-products. It has been clearly demonstrated that attack on pMAOS by primary amine nucleophiles proceeds not just at the NHS-activated methacrylate carbonyl, but also at the succinimidyl carbonyls. This ring-opening reaction is seen to be slightly favoured over the NHS-displacement reaction. This type of NHS ring-opening has been previously reported as a reasonably facile reaction, and it is thought to be so pronounced in the pMAOS system due to steric hindrance at the methacrylate carbonyl centre, presumably due at least in part to the polymer conformation in solution.

The resulting ring-opened hydroxamate moieties were shown to be displaced from the backbone by subsequent nucleophilic attack. However, this displacement was seen to occur not just through intermolecular attacks by primary amine nucleophiles, but also by intramolecular attack by neighboring amide moieties to form *N*-substituted glutarimide moieties. This glutarimide-forming reaction was shown to be unavoidable, proceeding at an equal rate to the intermolecular attack at 70 °C, for example. The copolymer products typically displayed very poor water solubility.

It is not known why these reactions have not been reported to date. It is possible that the size of the polymer chains has a significant effect in terms of steric hindrance around the NHS-activated carbonyls. The idea that polymer length may have an important influence on pMAOS conformation in solution and therefore the way that this polymer reacts with amine nucleophiles is purely speculative, and has yet to be further investigated.

The Godwin *et al.* paper[60] presents IR data but no NMR data, and the work described in Section 4.3.2.7 shows that the formation of copolymers **4.3** and **4.6** would not be easily detected in experiments monitored by IR spectroscopy alone. It is not known whether the polymeric products reported in this paper were fully characterised by NMR spectroscopy.

As an alternative to pMAOS, pNPMA was investigated. The use of this polymer, however, was also precluded by the formation of glutarimide moieties at levels greatly exceeding those seen with pMAOS. It is not known how universal this phenomenon of glutarimide formation is with poly(activated methacrylate) homopolymers, but these results suggest that the problem may be encountered with other activated polymeric precursors of this type.

Surprisingly, pMAC chloride showed much less glutarimide formation, though the synthesis of this material was thought to be hampered by hydrolysis, and the $\bar{M}_n$ of this material (which was likely to be much lower than the M_{target}) was not measured. This result therefore needs to be corroborated with pMAC of high quality, though the hydrolytic lability of this polymer may limit its application in the synthesis of polymer-drug constructs of type **1.1**.

CHAPTER 5

AIMS REVISITED

As has already been outlined in Section 1.4, at the beginning of the work described in this thesis, the aims of the work were defined as follows:

1) to further investigate and optimise the synthesis of DVB;

2) to extend the brief SAR investigation involving the methoxy and hydroxyvariolin series to the deoxy series;

3) to select the most suitable analogues for further development into polymer-drug conjugates, and acylate these analogues with the GFLG tetrapeptide biolinker; and,

4) to use any tetrapeptide-variolin conjugates successfully synthesised in the synthesis of polymer-drug conjugates from pMAOS.

The first of these aims was successfully fulfilled, with the overall yield for the six-step DVB synthesis being improved from 5.2% to 25%. The experimental details for this improvement are given in Chapter 2.

The second aim was also successfully fulfilled, with the *N,N*-dimethylaminopropaneamine moiety being selectively incorporated into DVB at either the C(9) and/or the C(2') positions. A 2'-methoxy analogue and two 9-alkyldiamine analogues were also synthesised. The biological data obtained for the deoxyvariolin analogues closely resembled that obtained by Anderson for the analogous compounds in the methoxy- and hydroxyvariolin series'. This work is described in Chapter 3.

The third objective, synthesis of variolin-tetrapeptide conjugates, was met with only partial success. By far the most bioactive analogue (at least in terms of the in-house P388 murine leukaemia bioassay) was DVB, but efforts to synthesise a tetrapeptide conjugate of this compound were unsuccessful. Experiments carried out with the 2'-SMe deoxyvariolin analogue **2.16** and the *N*(9a)-PMB DVB analogue **2.27** helped to shed some light onto this troublesome reactivity. Specifically, it was found that N(9a) is acylated more rapidly than N(2'a), but that the resulting *N*(9a)-acyl linkage is of inadequate stability to survive both the Fmoc-deprotection protocols and the polymer-drug synthetic protocols being employed in this work.

Having established that *N*(9a)-acylation gave compounds of insufficient stability, attention was turned towards acylating DVB at the N(2'a) position. Attempts at acylating N(2'a) of DVB quantitatively with Fmoc-glycine acid chloride were unsuccessful. It is not known why this reaction would not go to completion, though interference by water contamination can be ruled out. Purification of the resulting mixture of variolin compounds was prohibitively difficult.

N(9a)-PMB DVB analogue **2.27** was acylated by Fmoc-glycine acid chloride at N(2'a) and the product purified (albeit in low yield), but it was found that attempted PMB-deprotection using the established protocols (involving stirring in neat triflic acid) resulted in cleavage of the *N*(2'a)-acyl linkage.

In order to circumvent the difficulties encountered in the acylation of N(9a) and/or N(2'a) of DVB, deoxyvariolin analogue **3.23** was instead chosen for development into a polymer-drug construct. While possessing roughly an order of magnitude lower bioactivity than DVB, this analogue possessed only one primary aliphatic nucleophilic amine. Conjugation (and subsequent Fmoc-deprotection) of this analogue with the tetrapeptide biolinker proceeded without complication to give conjugate **3.26** in a total of six steps with 23% overall yield (from commercially available starting materials.

With conjugate **3.26** in hand, the fourth aim of the project, namely, synthesis of polymer-drug conjugates from pMAOS, was addressed. Despite the numerous literature accounts of the aminolysis of pMAOS, attempts to synthesise a HPMA-based polymer drug conjugate of type **1.1** incorporating deoxyvariolin analogue **3.23** from pMAOS proved unsuccessful. Subsequent investigations involving only 1A2P as nucleophile uncovered two unforeseen side reactions. Specifically, attack by 1A2P at the succinimidyl carbonyls was resulting in ring-opening of the NHS groups (with 60-65% ring-opening typically being observed), and a subsequent attack by

HPMA amide nitrogens on neighbouring hydroxamate-activated esters was leading to *N*-substituted glutarimide formation. This glutarimide formation was shown to be a surprisingly facile and unavoidable reaction. Furthermore, the polymeric products displayed poor water solubility. These two side reactions ultimately precluded the synthesis of polymer-drug constructs from pMAOS.

One solution to the problem of glutarimide formation seen with the aminolysis of poly(methacrylic activated esters) may be found through the use of *N*-methylated nucleophiles, such as *N*-methyl 1A2P. However, the biological profiles of the resulting polymers, for example, poly[*N*-methyl-*N*-(2-hydroxypropyl)methacrylamide], are yet to be determined.

A more reasonable approach would be to simply employ the copolymerisation protocols that have been shown to be successful in various publications in recent decades (see Section 1.2.3.1 for a discussion of these protocols). Specifically, the well-established PK1-type synthetic protocols could be employed to give an HPMA-based copolymer carrying analogue **3.23**.

Alternatively, this polymer-drug conjugate could be made by the copolymerisation of HPMA with a methacryloyl-tetrapeptide-variolin monomer (which should be readily accessible via the acylation of **3.26** with methacryloyl chloride). Importantly, deoxyvariolin analogue **3.23** would be expected to be stable under the polymerisation conditions.

Incorporation of the more bioactive DVB onto a polymer backbone is desirable. Of the two possible points of attachment, the N(2'a) position of DVB would give the more stable conjugate. Synthesis of pHPMA-based prodrugs of this type would first necessitate the synthesis of an appropriately acylated DVB analogue. Given that the pMAOS methodology is seriously flawed, a copolymerisation involving HPMA and a methacryloyl-tetrapeptide-DVB conjugate would be necessary. Synthesis of the appropriate DVB-bearing monomer would initially require the efficient synthesis of *N*(2'a)-acylated DVB analogues. Given the difficulties encountered with the acylation of DVB, this goal is probably best achieved through the acylation of an *N*(9a)-protected DVB analogue with Fmocglycine acid chloride. PMB has already been shown to be an unsuitable protecting group for this synthetic route, given the harsh conditions needed for deprotection. Alternative protecting group strategies therefore need to be investigated. The allyloxycarbonyl (Alloc) group is one such possibility.

Finally, the low stability of the deoxyvariolin *N*(9a)-acyl linkage, particularly in acidic solutions, could be capitalised on in the synthesis of polymer-drug constructs incorporating DVB bound via

the N(9a) position. The acidic environment of tumour interstitia and of lysosomes would be expected to facilitate the liberation of DVB bound in this manner from the polymer backbone. Such constructs could conceivably be synthesised by the well-established PK1-type synthetic protocols, employing DVB as the nucleophilic drug. An EDCI-mediated coupling between DVB and an HPMA/methacryloyl-tetrapeptide copolymer is a variation on this theme that should also give the desired product. The polymer product from these two analogous synthetic routes would give a construct from which DVB would be liberated via cathepsin-mediated *and* acid-catalysed hydrolysis.

CHAPTER 6

EXPERIMENTAL

6.1 General Methods

Nuclear Magnetic Resonance Spectroscopy

All proton detected NMR spectra were obtained on a Varian Inova 500 spectrometer at 23 °C, operating at 500 MHz. Carbon detected NMR spectra were recorded on a Varian Unity 300 spectrometer at 23 °C and operating at 75 MHz. Other NMR experiments described in this thesis *viz* COSY and the reverse detected HSQC and CIGAR experiments were obtained on the Inova 500 spectrometer at 500 MHz. Chemical shifts in this thesis are described in parts per million (ppm), on the δ scale, and were referenced to appropriate solvent peaks: $CDCl_3$ referenced to $Si(CH_3)_4$ at δ_H 0 ppm (^1H) and $CHCl_3$ at δ_C 77.0 ppm (^{13}C); d_4-methanol referenced to CHD_2OD at δ_H 3.30 ppm (^1H) and CD_3OD at δ_C 49.3 ppm (^{13}C); and d_6-DMSO referenced to $CD_3(CHD_2)SO$ at δ_H 2.50 ppm (^1H) and $(CD_3)_2SO$ at δ_C 39.6 ppm (^{13}C). ^1H NMR spectra were recorded using an acquisition time (AT) of 0.878 s and a relaxation delay (D1) of 2 s. COSY experiments were typically recorded using an AT of 0.137 s and a D1 of 1.0 s. HSQC-DEPT experiments with the Pulsed Field Gradient system were generally run with an AT of 0.133 s, a D1 of 1.0 s and $J_{C\text{-}H}$ of 130-165 Hz. CIGAR experiments with the Pulsed Field Gradient system were run with an AT of 0.303 s, a D1 of 1.0 s, J = 140 Hz and a $^nJ_{CH}$ of 8.0 Hz.

High Pressure Liquid Chromatography

The High Pressure Liquid Chromatography (HPLC) work described in this thesis was performed on a Shimadzu VP system. The complete setup involved a Shimadzu LC-10AC VP liquid chromatograph coupled to an SIL-10A VP autoinjector, a CTO-10A VP column oven set to 40 °C, and a SPD-M10A VP diode array detector. This system was controlled by Shimadzu CLASS-VP (Version 5.023) software. A Shimadzu degasser (DGU-14A) was utilised for the degassing of all solvents used in this machine.

The column used was a Phenomenex ODS(3) column (250 × 4.6 mm, 60 Å, 5 μm APD, 1 mL/min).

The solvents used were mixtures of either acetonitrile (BDH HiperSolv™ 'Far UV' grade) or methanol (BDH HiperSolv™ grade) with water (purified using a MilliQ deionising system). The aqueous phase was acidified with 0.05% TFA (Scharlau, synthesis grade).

The standard gradient elution protocols are given below:

Time (min)	B conc. (%)*
0	10
2	10
14	75
24	75
26	100
30	100
32	10
40	10

* This refers to the concentration of acetonitrile in the eluant

Gel Permeation Chromatography

GPC analysis of pMAOS samples was conducted at Ian Wark Laboratories, CSIRO, Clayton, Victoria, Australia. The samples were dissolved in DMF and filtered prior to the analysis. They were analysed using a series of four Styragel columns HT2, HT3, HT4 and HT5 and an oven temperature of 80 °C. The eluants used were DMF and 0.05 M LiBr at a flow rate of 1.0 mL/min. A Dawn EOS light scattering detector with Optilab DSP interferometer (both set at 690 nm) was used.

Mass Spectrometry

Mass spectrometric analyses were performed on a Micromass LCT spectrometer equipped with an electrospray ionisation (ESI) probe. Samples were analysed at a probe voltage of 3200 V at 150 °C with a nebuliser gas flow of 160 L/hr and desolvation gas flow of 520 L/hr with the source temperature at 80 °C. The cone voltage was typically 20 V. The solvent flow from a syringe pump in direct injection mode was 20 μL/min.

UV-Vis Spectroscopy

UV-Vis measurements were taken on a Hewlett Packard HP 8452A Diode Array Spectrometer. Samples were placed in 10 mm quartz glass cuvettes (Starna).

IR spectroscopy

IR measurements were taken on a Shimadzu FTIR-8201 PC Spectrometer. Spectra of solids were obtained by the diffuse reflectance method using KBr. Spectra of oils were obtained in a solution of $CDCl_3$ using a CaF_2 solution cell.

Melting Points

Melting points were obtained on a Reichert microscope hot-stage and are uncorrected.

Column Chromatography

Analytical thin layer chromatography (TLC) was conducted on aluminium-backed Merck Kieselgel KG60F_{254} silica sheets (0.2 mm thickness). Plates were visualised by both short- and long-wavelength UV light. For compounds possessing the pyrido[3',2':4,5]pyrrolo[1,2-c]pyrimidine core, long-wave UV light was particularly useful. Staining was occasionally carried out using either potassium permanganate dip (3 g $KMnO_4$, 20 g K_2CO_3 and 5 mL 5% aq NaOH in 300 mL H_2O) or phosphomolybdic acid dip (10% w/v phosphomolybdic acid in EtOH).

Flash chromatography was routinely carried out using Merck silica 60 (40-63μ) following the procedure of Still and coworkers.[133] Where stated, neutralised silica was prepared by stirring the silica as a slurry in CH_2Cl_2 with 0.1 v/w equivalents of triethylamine, then, following loading into a column, washing with 3-5 column volumes of the eluant. Reversed phase C18 chromatography was carried out using Bakerbond C_{18} (40 μm preperative LC packing), which was recycled by successive washes with 1% TFA/DMSO, MeOH and CH_2Cl_2. Bakerbond spe™ Octadecyl (C_{18}) disposable solid phase extraction columns (40 mm APD, 60 Å) of a 500 mg/3 mL size were also occasionally employed for small samples. DIOL chromatography was carried out with J.T.Baker DIOL (40 μm prep LC packing), which was recycled by successive washes with 1% TFA/DMSO, MeOH and CH_2Cl_2. Preparative size-exclusion chromatography (SEC) was carried out using Sephadex LH-20 (Pharmacia Biotech AB). GPC analyses were performed at the Key Centre for Polymer Colloids, University of Sydney, Australia by Uma Adash, using a series of four Styragel columns HT2, HT3, HT4 and HT5 and an oven temperature of 80 °C. The eluant was DMF + 0.05 M LiBr at a flow rate of 1.0 mL/min. A Dawn EOS light scattering detector with Optilab DSP interferometer (both set at 690 nm) was used.

Solvents were all of commercial grade and distilled once in glass distillation apparatus except MeOH, which was distilled twice. Hexanes consisted of the fraction boiling at 50-70 °C. 'Flash' columns were run under oxygen-free N_2 gas pressure (0.5 kPa).

Following chromatography, synthetic products were analysed by ^{1}H NMR spectroscopy in order to ascertain their purity. Unless otherwise stated, this purity was close to 100%.

P388 (Murine Leukaemia) *in vitro* Bioassay

Compounds were assayed for cytotoxicity where applicable using the in-house P388 MTT antitumour assay, with samples being submitted as solutions in either MeOH or 2:1 $MeOH/CH_2Cl_2$. The antitumour assay is the most sensitive assay for cytotoxicity. It consists of a 2-fold dilution series of the sample of interest followed by incubation for 72 h with P388 cells. The concentration of sample required to reduce the P388 cell growth by 50% (compared to control cells) is determined using the absorbance values obtained when the yellow MTT tetrazolium dye is reduced by healthy cells to the purple MTT formazan. The result is expressed as the IC_{50}, usually either in ng/mL or μM.

Solvents and Reagents

Reagents and solvents used in reactions were purified according to well-established procedures.[134] All technical grade solvents were distilled prior to use. Methanol was distilled twice. Ethanol stabiliser was removed from chloroform by washing with water then distilling from calcium chloride. Tetrahydrofuran and diethyl ether were distilled from sodium benzophenone ketyl immediately prior to use. Toluene, dichloromethane, pyridine and triethylamine were distilled from calcium hydride. Acetic anhydride was distilled from phosphorous pentoxide. 1,2-Dichloroethane was distilled from phosphorous pentoxide and stored over 4 Å sieves under an atmosphere of nitrogen. Acetyl chloride was distilled from quinoline (10% v/v) immediately prior to use. Both *N*,*N*-dimethylformamide and dimethylsulfoxide were sequentially dried over two batches of freshly activated 4 Å sieves (2 × 24 h), before finally being stored over a third batch of freshly activated 4 Å sieves. To remove residual dimethylamine, DMF was evacuated (~0.1 mm Hg) for at least 15 min prior to use. Dry methanol was obtained by distillation from $Mg(OMe)_2$ and stored under an atmosphere of nitrogen. Solutions of *n*-BuLi in hexanes were titrated in diethyl ether with 2-butanol, using 1,10-phenanthroline as an indicator. *m*-Chloroperbenzoic acid was washed with pH = 7.5 phosphate buffer and recrystallised from dichloromethane. Concentrated aqueous ammonia solution was of analytical grade, and was stated to contain ~25% ammonia, density = 0.91 g/mL.

Water-sensitive reactions were performed in flame-dried glassware under an atmosphere of dry argon or nitrogen. Reaction temperatures refer to the external bath or oven temperature. Where

stated, "concentrated *in vacuo*" will generally refer to removal of solvents on a Buchi rotary evaporator, followed by removal of traces of solvent using a high vacuum pump.

6.2 Experiments Described in Chapter 2

4-Iodo-2-(methylsulfanyl)pyrimidine 2.1[135]

4-Chloro-2-(methylsulfanyl)pyrimidine (14.00 g, 87.2 mmol) was added to hydroiodic acid (100 mL, 731 mmol) with vigorous stirring, and left to stir at rt for 3 days. Large lumps were regularly broken up using a glass rod during this time. The suspension was filtered, and the precipitate washed with water. The brown solid was then dissolved in CH_2Cl_2 (200 mL) and washed with sat aq $NaHCO_3$ solution (200 mL), 5% aq $Na_2S_2O_3$ solution (200 mL), sat brine solution (200 mL), dried with Na_2SO_4 and filtered. The solvent was removed *in vacuo*, and the crude material thus obtained recrystallised from hexanes to afford the pure iodide as large white needles (15.49 g, 70% yield). Concentration *in vacuo* of the mother liquor followed by recrystallisation of the residues from hot hexanes gave more of the above (2.12 g, 80% total yield).

Mp: 49-50 °C (hexanes) (lit 52-53 °C).[135]

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.54 (s, 3H, SCH_3), 7.41 (d, J = 5.4 Hz, 1H), 8.00 (d, J = 4.9 Hz, 1H).

2-Chloronicotinoyl chloride 2.3[136]

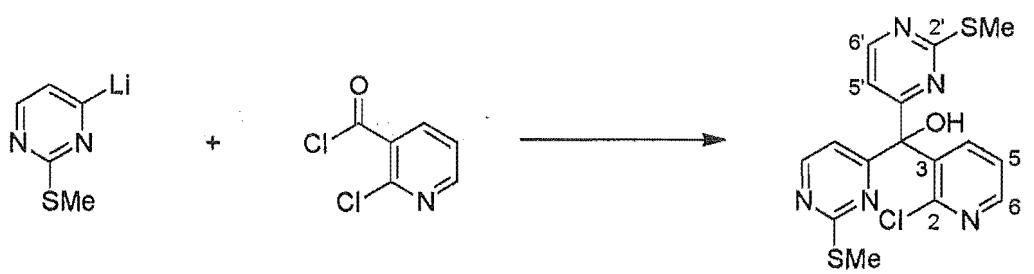
2-Chloronicotinic acid (4.02 g, 26 mmol), thionyl chloride (10.0 mL, 0.137 mmol) and dry DMF (0.2 mL, 2.6 mmol) were stirred at reflux under argon for 24 h. After cooling, thionyl chloride

and DMF were removed *in vacuo*, and the product purified by Kugelrohr distillation (120 °C, 0.03 mm Hg) to afford the acid chloride as a white crystalline solid (4.45 g, 99% yield). The solid was stored under argon and used within 1 day of distillation.

Mp: 38-41 °C, sweats at 34 °C (lit 39-44 °C).[137]

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.44 (dd, J = 4.9, 7.8 Hz, 1H), 8.40 (dd, J = 2.0, 8.3 Hz, 1H), 8.59 (dd, J = 2.0, 4.9 Hz, 1H).

(2-Chloropyridin-3-yl)bis-[2-(methylsulfanyl)pyrimidin-4-yl]methanol 2.4



a) Non-Barbier protocols:[87]

The reactor was charged with iodide **2.1** (9.23 g, 7.9 mmol) and THF (350 mL), and cooled to -95 °C (MeOH/N_2). A solution of *n*-BuLi in hexanes (1.65 M, 22.18 mL, 36.6 mmol) was carefully added to the stirred solution over 45 min, while maintaining the solution at -95 °C. The reactor coil was carefully washed with THF (5 mL), and the solution stirred at -95 °C for 30 min. To the deep red/brown solution was added a solution of acid chloride **2.3** (2.16 g, 12.3 mmol) in THF (20 mL) over 40 min. After 4 h at -95 °C the reaction was left stirring for 10 h, during which time it warmed slightly to -93 °C. The solution was then allowed to warm to rt and washed with sat aq NH_4Cl and extracted with EtOAc (500 mL, 250 mL, 100 mL). The organic extractions were pooled, filtered, concentrated *in vacuo* to a volume of ~400 mL, washed with sat brine solution (400 mL), dried with $MgSO_4$, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by repeated flash chromatography on silica, eluting with 1:1 EtOAc/hexanes, to afford the triaryl alcohol as an orange foam [1.32 g, ~95% pure (as determined by ^{1}H NMR), 26% yield].

b) Barbier protocols:

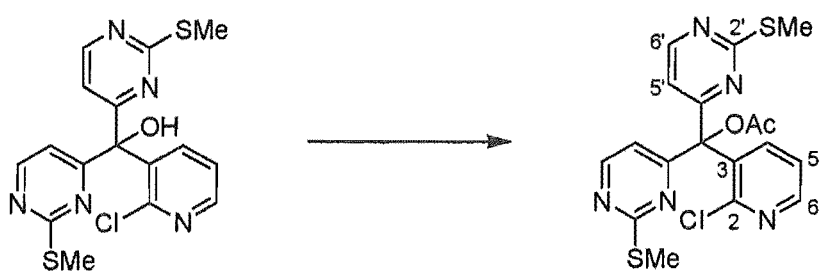
A solution of iodide **2.1** (1.58 g, 6.3 mmol) in THF (17 mL) was transferred via cannula to a pre-cooled solution of acid chloride **2.3** (500 mg, 2.8 mmol) in THF (8 mL) at -96 °C. A solution of *n*-BuLi in hexanes (1.70 M, 3.68 mL, 6.3 mmol) was then added dropwise over 10 min with vigorous stirring. The dark red/brown solution was stirred at -96 °C for a further 2 h then methanol (400 μL) was added over 1 min. The deep red solution was stirred at rt for 2 h, then diluted with CH_2Cl_2 (75 mL), washed with sat aq $NaHCO_3$ solution (100 mL), sat brine solution (100 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was dissolved in benzene (15 mL), MnO_2 (1.0 g 11.5 mmol) added, and the black suspension heated at reflux for 2 h. Filtration through a celite plug followed by concentration *in vacuo* gave an oil which was purified by flash chromatography on silica, eluting with 50% EtOAc/hexanes, to afford the triaryl methanol as an orange solid (521 mg, 95% pure, 40% yield).

Mp: 55-58 °C (lit 54-58 °C).[87]

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.49 (s, 3H, SC*H*$_3$), 6.33 (s, 1H, O*H*), 7.17 (dd, J = 4.4, 7.8 Hz, 1H, H-5), 7.23 (dd, J = 2.0, 7.8 Hz, 1H, H-4), 7.40 (d, J = 4.9 Hz, 1H, H-5'), 8.38 (dd, J = 2.0, 4.4 Hz, 1H, H-6), 8.57 (d, J = 4.9 Hz, 1H, H-6').

(2-Chloropyridin-3-yl)bis-[2-(methylsulfanyl)pyrimidin-4-yl]methyl acetate 2.5

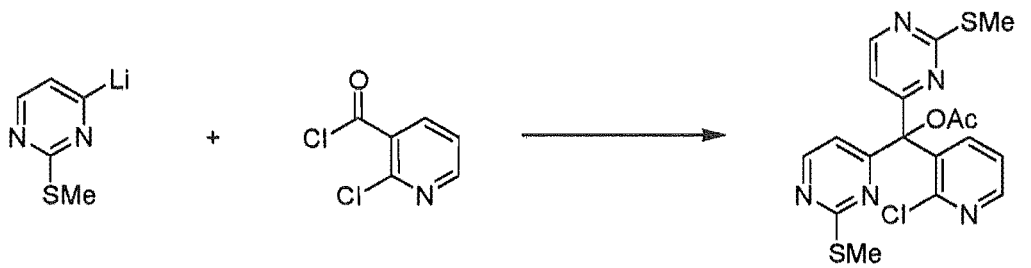
a) From triaryl alcohol 2.4:



To a solution of triaryl alcohol **2.4** (497 mg, 1.27 mmol), DMAP (10.0 mg, 82 μmol) and TEA (265 μL, 1.90 mmol) in CH_2Cl_2 (6 mL) was added acetic anhydride (239 μL, 2.53 mmol) and the solution heated at reflux for 20 h. The solution was allowed to cool to rt and concentrated *in vacuo*. The crude material thus obtained was purified by repeated flash chromatography on

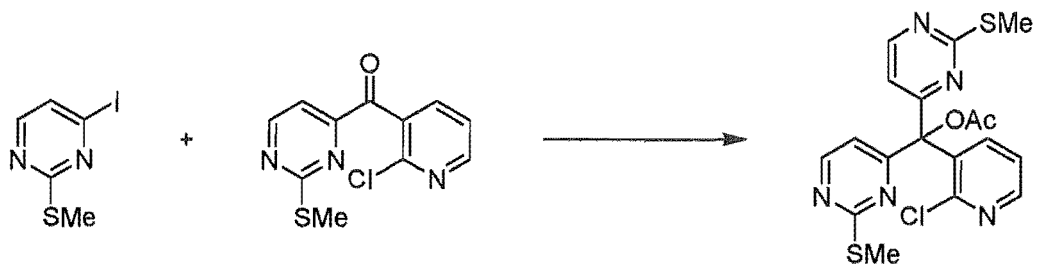
silica, eluting with 1:1 EtOAc/hexanes, to afford the title compound as an off-white amorphous solid (506 mg, 92% yield).

b) Non-Barbier lithiation protocols:[87]



The reactor was charged with iodide **2.1** (2.00 g, 7.9 mmol) and THF (100 mL), and cooled to -95 °C (MeOH/N_2). A solution of *n*-BuLi in hexanes (1.58 M, 5.03 mL, 7.9 mmol) was carefully added to the vigorously stirred solution over 15 min, while maintaining the solution at -95 °C. After 30 min, a solution of acid chloride **2.3** (465 mg, 2.6 mmol) in THF (0.47 mL) was added to the dark mixture over 7 min. After 4.5 h at -95 °C, the reaction was quenched with AcCl (566 μL, 7.9 mmol) and allowed to warm overnight. The solution was diluted with CH_2Cl_2 (100 mL), washed with sat aq $NaHCO_3$ solution (200 mL), sat brine solution (200 mL), dried with $MgSO_4$, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by flash chromatography on silica, eluting with 1:1 EtOAc/hexanes, to afford the triaryl acetate as an orange foam (724 mg, 95% pure, 60% yield).

c) Grignard arylation of ketone ***2.9****:*[91]



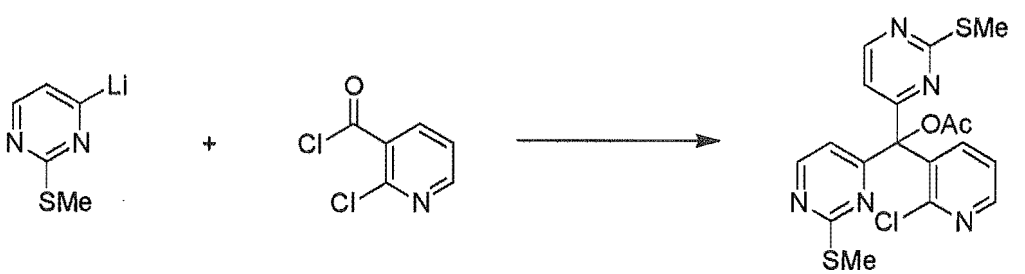
To a stirred solution of iodide **2.1** (743 mg, 2.95 mmol) in toluene (18 mL) at 0 °C was added a solution of *i*-PrMgCl in diethyl ether (1.36 M, 2.17 mL, 2.95 mmol) dropwise over 8 min. The

resulting brown solution was stirred at 0 °C for 1 h, then transferred via cannula over 5 min to a similarly cooled solution of ketone **2.9** (313 mg, 1.18 mmol) in toluene (12 mL), and the solution stirred at 0 °C for 15 min. AcCl (294 μL, 4.12 mmol) was added over 1 min, and the solution allowed to warm to rt overnight. At this time sat aq $NaHCO_3$ solution (30 mL) was added and the biphasic solution extracted with EtOAc (3 × 15 mL). The organic extractions were combined, dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was subjected to repeated flash chromatography on silica, eluting with 1:1 EtOAc/hexanes, to give the triaryl acetate in low purity as an orange/brown oil (147 mg, 50% pure, 14% yield).

d) Barbier lithiation protocols from ketone 2.9:[91]

To a vigorously stirred solution of iodide **2.1** (2.16 g, 8.6 mmol) and ketone **2.9** (1.14 g, 4.3 mmol) in THF (25 mL) at -78 °C was added a solution of *n*-BuLi in hexanes (1.52 M, 5.64 mL, 8.57 mmol) over 15 min. The brown solution was stirred at -78 °C for 15 min, AcCl (1.22 mL, 17.1 mmol) added dropwise over 2 min and the dark red solution stirred at rt for 3 h. The solution was diluted with CH_2Cl_2 (100 mL), washed with sat aq $NaHCO_3$ solution (100 mL), sat brine solution (100 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo* to give a dark orange/brown oil. This crude material was purified by flash chromatography on silica, eluting with 1:1 EtOAc/hexanes, to give the triaryl acetate, contaminated by a small amount of the biaryl acetate, as an orange foam (1.23 g, 90% pure, 59% yield).

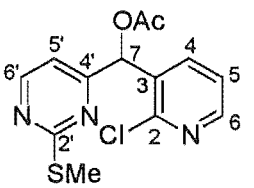
e) Barbier lithiation protocols from acid chloride 2.3 (with biaryl acetate 2.15 formation):



To a vigorously stirred solution of acid chloride **2.3** (163 mg, 0.93 mmol) and iodide **2.1** (700 mg, 2.8 mmol) in THF (9 mL) at -78 °C was added a solution of *n*-BuLi in hexanes (1.52 M, 1.83 mL, 7.93 mmol) dropwise over 5 min. The dark brown solution was stirred at

-78 °C for 30 min, then acetyl chloride (330 μL, 4.6 mmol) added over 2.5 min. The deep red solution was stirred at rt for 3 h, then diluted with CH_2Cl_2 (30 mL), washed with sat aq $NaHCO_3$ solution (20 mL), sat brine solution (20 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by flash chromatography on silica, eluting with 1:1 EtOAc/hexanes, to give an inseparable mixture of the triaryl acetate and biaryl acetate in a ratio of 10:1 (291 mg, 90% pure, 65% yield of the triarylacetate).

Biaryl acetate **2.15** had the following spectroscopic properties (some signals, as reported in the literature, are coincident with the triarylacetate signals):[91]



^{1}H NMR (500 MHz, $CDCl_3$): δ 2.22 (s, 3H, $COCH_3$), 2.45 (s, 3H, SCH_3), 7.04 (s, 1H, H7), 7.11 (d, J = 5.2 Hz, 1H, H-5'), 7.27 (coincident, 1H, H-5), 7.83 (dd, J = 1.7, 8.1 Hz, 1H, H-4), 8.39 (coincident, 1H, H-6), 8.53 (d, J = 4.8 Hz, 1H, H-6').

*f) Barbier lithiation protocols from acid chloride **2.3** (without biaryl acetate **2.15** formation):*

A solution of acid chloride **2.3** (1.04 g, 5.9 mmol) in THF (10 mL) was transferred via cannula to a pre-cooled solution of iodide **2.1** (3.30 g, 13.1 mmol) in THF (40 mL) at -96 °C. A solution of *n*-BuLi in hexanes (1.60 M, 8.17 mL, 13.1 mmol) was then added dropwise over 20 min with vigorous stirring. The dark red/brown solution was stirred at -96 °C for a further 2h then acetyl chloride (2.11 mL, 30 mmol) was added over 5 min. The deep red solution was stirred at rt for 16 h, then diluted with CH_2Cl_2 (100 mL), washed with sat aq $NaHCO_3$ solution (200 mL), sat brine solution (150 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by repeated flash chromatography on DIOL, eluting with 10-30% EtOAc/hexanes,* to give the triaryl acetate as an orange solid (1.30 g, 98% pure, 50% yield).

* Purification could also be carried out on silica, eluting with 1:1 EtOAc/hexanes, to give similar yields.

Mp: 58-61 °C.

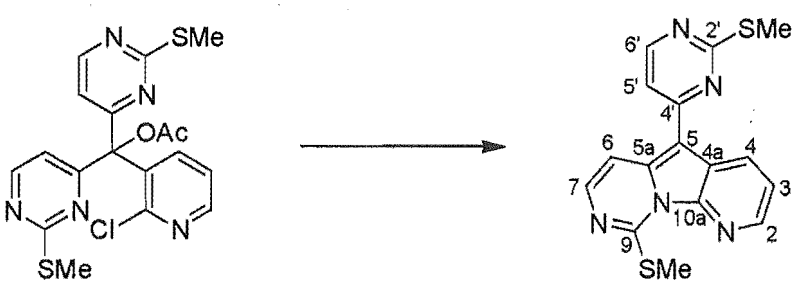
^{1}H NMR (500 MHz, $CDCl_3$): δ 2.30 (s, 3H, $COCH_3$), 2.39 (s, 6H, SCH_3), 7.26 (dd, J = 4.9, 7.8 Hz, 1H, H-5), 7.30 (d, J = 4.9 Hz, 2H, H-5'), 7.89 (dd, J = 1.7, 8.1 Hz, 1H, H-4), 8.39 (dd, J = 2.0, 4.9 Hz, 1H, H-6), 8.51 (d, J = 4.8 Hz, 2H, H-6').

^{13}C NMR (75 MHz, $CDCl_3$): δ 14.0 (SCH_3), 21.3 ($COCH_3$), 85.9 ($C(Ar)_3OAc$), 114.7 (C-5'), 121.1 (C-5), 134.0 (C-3), 141.3 (C-4), 149.2 (C-6), 150.3 (C-2), 157.4 (C-6'), 166.1 (C-4'), 168.6 ($COCH_3$), 172.6 (C-2').

IR (KBr): υ_{max} 1757, 1549, 1400, 1348, 1207, 1086, 1061, 903.

HRMS (ESI): Calcd for $C_{18}H_{17}{}^{35}ClN_5O_2{}^{32}S_2$ (MH^+) *m/z* = 434.0512, found *m/z* = 434.0512.

9-(Methylsulfanyl)-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.6



*a) From triaryl acetate **2.5** (free of biaryl acetate **2.15** contamination):*[91]

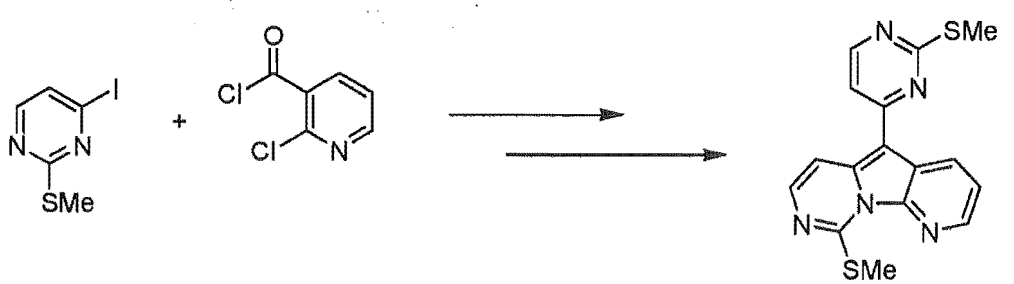
Triaryl acetate **2.5** (745 mg, 1.72 mmol) was heated with TFA (264 μL, 3.43 mmol) and triethylsilane (2.19 mL, 13.7 mmol) in 1,2-dichloroethane (4 mL) at 100°C for 3 d. The orange solution was diluted with CH_2Cl_2 (350 mL), washed with sat aq $NaHCO_3$ solution (300 mL), sat brine solution (300 mL), dried with $MgSO_4$, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by repeated flash chromatography on silica, eluting with 37% EtOAc/hexanes, to give the variolin core as a yellow microcrystalline solid (424 mg, 95% pure, 69% yield).

*b) From triaryl acetate **2.5** (with the biaryl acetate **2.15** contamination):*

A 4:1 mixture of triaryl acetate **2.5** and biaryl acetate **2.15** (1.42 g, 2.6 mmol of the triaryl acetate) was heated with TFA (503 μL, 6.53 mmol) and triethylsilane (3.04 mL, 26.1 mmol) in

1,2-dichloroethane (7 mL) at 100°C for 3 d. The orange solution was allowed to cool to rt then diluted with CH_2Cl_2 (100 mL), washed with sat aq $NaHCO_3$ solution (100 mL), sat brine solution (100 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The dark orange/brown oil thus obtained was dissolved in THF (250 mL), 1M aq NaOH (250 mL) added, and the resulting brown solution stirred at rt for 1 h. Extraction with CH_2Cl_2 (500 mL) gave a red/brown organic solution that was washed with sat brine solution (500 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo* to give an orange/brown oil. The crude material thus obtained was purified by flash chromatography on neutralised silica, eluting with 5% EtOAc/CH_2Cl_2 (0.1% TEA), to give the variolin core as an orange microcrystalline solid (642 mg, 72% yield).

*c) From acid iodide **2.1** and chloride **2.3** (without purification of the triaryl acetate intermediate **2.5**):*



A solution of iodide **2.1** (3.16 g, 12.6 mmol) in THF (10 mL) was transferred via cannula to a pre-cooled solution of acid chloride **2.3** (1.00 g, 5.7 mmol) in THF (40 mL) at -96 °C. A solution of *n*-BuLi in hexanes (1.58 M, 7.94 mL, 12.6 mmol) was added dropwise over 20 min with vigorous stirring. The dark red/brown solution was stirred at -96 °C for a further 2h then acetyl chloride (2.04 mL, 28.5 mmol) was added over 5 min. The deep red solution was stirred at rt for 1 h then diluted with CH_2Cl_2 (100 mL), washed with sat aq $NaHCO_3$ solution (100 mL), sat brine solution (100 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The dark orange/brown oil thus obtained was transferred to a Young's tube and left at reduced pressure (0.03 mm Hg) for 16 h. To this crude material was then added 1,2-dichloroethane (10 mL), TFA (879 μL, 11.4 mmol) and triethylsilane (7.27 mL, 45.6 mmol), and the resulting dark red/brown solution incubated at 100 °C for 3 d. The solution was allowed to cool to rt then diluted with CH_2Cl_2 (400 mL), washed with sat aq $NaHCO_3$ (400 mL), sat brine solution (400 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified

by flash chromatography on silica, eluting with 10% EtOAc/ CH_2Cl_2, to give the variolin core as a yellow microcrystalline solid (985 mg, 98% pure, 50% yield over 2 steps).

Mp: 209-211 °C [lit 210-11 °C (MeOH/1,2-dichloroethane)].[87]

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.68 (s, 3H, 2'-SC*H*$_3$), 2.74 (s, 3H, 9-SC*H*$_3$), 7.35 (d, J = 5.4 Hz, 1H, H-5'), 7.51 (dd, J = 4.6, 8.1 Hz, 1H, H-3), 7.82 (d, J = 6.3 Hz, 1H, H-7), 8.06 (d, J = 6.8 Hz, 1H, H-6), 8.52 (d, J = 5.4 Hz, 1H, H-6'), 8.60 (dd, J = 1.7, 4.6 Hz, 1H, H-2), 8.64 (dd, J = 1.7, 8.1 Hz, 1H, H-4).

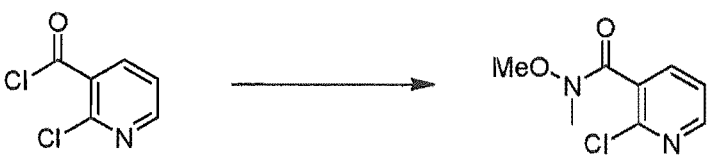
^{13}C NMR (75 MHz, $CDCl_3$): δ 14.3 (2'-SCH$_3$), 14.9 (9-SCH$_3$), 101.5 (C-5), 108.3 (C-6), 113.0 (C-5'), 120.71 (C-4a), 120.84 (C-3), 128.0 (C-4), 137.5 (C-5a), 139.9 (C-7), 141.8 (C-2), 143.0 (C-10a), 155.1 (C-9), 156.7 (C-6'), 160.9 (C-4'), 172.5 (C-2').

IR (KBr): υ_{max} 3140-2850 (series of weak bands), 1936. 1562, 1462, 1443, 1362, 1242, 1188, 964, 826.

HRMS (ESI): Calcd for $C_{16}H_{14}N_5{}^{32}S_2$ (MH^+) *m/z* = 340.0691, found *m/z* = 340.0692.

IC_{50} (P388): >37 μM

2-Chloro-*N*-methoxy-*N*-methyl-nicotinamide 2.8[91]

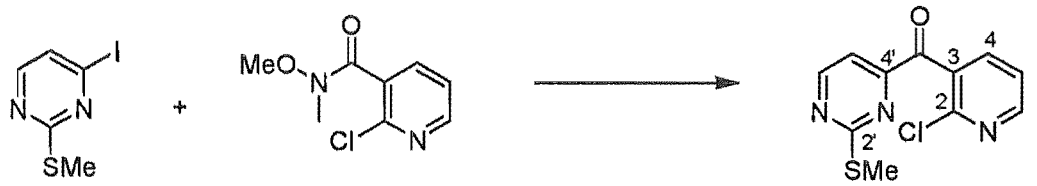


To a suspension of *N,O*-dimethylhydroxylamine hydrochloride (604 mg, 6.19 mmol) in THF (20 mL) was added TEA (1.71 mL, 12.4 mmol) and the white suspension stirred under N_2 at rt for 30 min. To this was added 2-chloronicotinoyl chloride (1.089 g, 6.19 mmol) and the suspension stirred at rt for 18 h. At this time the suspension was diluted with sat aq $NaHCO_3$ (40 mL) and the colourless solution extracted with CH_2Cl_2 (3 × 25 mL). The organic extractions were combined, dried with Na_2SO_4, filtered and concentrated *in vacuo* to afford the Weinreb's amide as a light brown amorphous solid (1.16 g, 93% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.41 (s, 3H, Me), 3.50 (s, 3H, Me), 7.29-7.34 (dd, J = 4.8, 7.1 Hz, 1H, Ar), 7.70 (d, J = 7.1 Hz, 1H, Ar), 8.46 (d, J = 3.8 Hz, 1H, Ar).

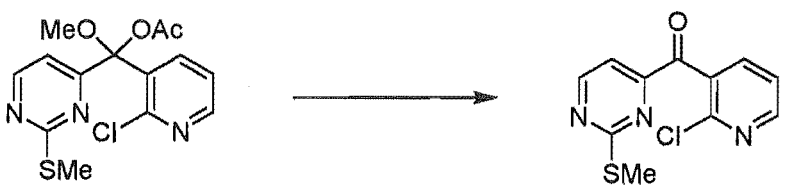
(2-Chloropyridin-3-yl)-[2-(methylsulfanyl)pyrimidin-4-yl]methanone 2.9

a) From Weinreb's amide 2.8:[91]



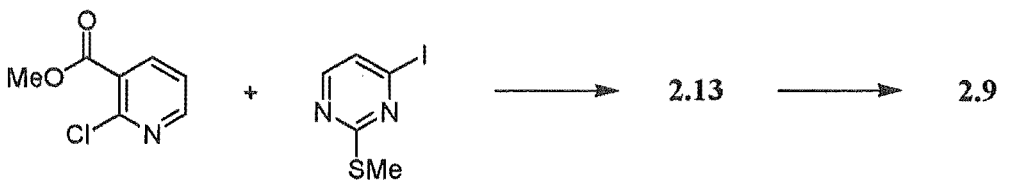
To a vigorously stirred solution of iodide **2.1** (1.81 g, 7.2 mmol) in toluene (10 mL) at -15 °C was added a solution of *i*-PrMgCl in diethyl ether (1.46 M, 3.93 mL, 7.2 mmol) dropwise over 10 min, and the resulting light brown solution stirred at -10 °C for 45 min. Into this was transferred, via cannula, a similarly cooled solution of Weinreb's amide **2.8** (1.15 g, 5.7 mmol) in THF (3 mL) dropwise over 10 min, and the resulting brown solution stirred at -5 °C for 1 h. The reaction was quenched with aq NH_4Cl (13 mL) and extracted with EtOAc (2 × 13 mL). The organic extractions were combined, dried with Na_2SO_4, filtered and concentrated *in vacuo*. The brown residues thus obtained were triturated with 3:1 diethyl ether/hexanes (2 × 5 mL, 5 × 2 mL) and dried *in vacuo* to afford the ketone as a light brown solid (1.14 g, 75% yield).

b) From quaternary species 2.13:



Quaternary species **2.13** (284 mg, 836 μmol) was dissolved in MeOH (100 mL), 1M aq NaOH solution (100 mL) added and the brown solution stirred at rt for 2 h. The brown solution was diluted with sat brine solution (100 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The organic extractions were combined, dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by flash chromatography on silica, eluting with 1:1 EtOAc/hexanes, to afford the ketone as an orange foam (170 mg, 77% yield).

*c) From methyl ester **2.12** (without purification of quaternary species **2.13**):*

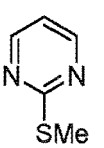


To a vigorously stirred and cooled solution of methyl ester **2.12** (370 mg, 2.16 mmol) and iodide **2.1** (1.087 g, 4.31 mmol) at -78 °C was added a solution of *n*-BuLi in hexanes (1.52 M, 2.84 mL) dropwise over 5 min. The dark brown mixture was stirred at this temperature for 30 min, then AcCl added dropwise over 1 min and the solution stirred at rt for 3 h. The solution was then poured slowly into 1 M aq NaOH (25 mL) at 0 °C with vigorous stirring, then stirred at rt for 2 h. The solution was diluted with CH_2Cl_2 (30 mL) to give a red/brown organic solution that was washed with sat brine solution (20 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by repeated flash chromatography on silica, eluting with 40% EtOAc/hexanes, to give a 3:1 mixture of the title compound **2.9** and contaminant **2.10**, shown below, as an orange foam (334 mg, ~70% yield of the ketone).

Mp: 98-100 °C [lit. 107-9 °C (benzene/hexanes)].[87]

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.38 (s, 3H, SCH_3), 7.41 (dd, J = 4.8, 7.5 Hz, 1H, H-5), 7.65 (d, J = 4.8 Hz, 1H, H-5'), 7.89 (dd, J = 2.0, 7.5 Hz, 1H, H-4), 8.57 (dd, J = 2.0, 5.2 Hz, 1H, H-6), 8.83 (d, J = 4.8 Hz, 1H, H-6').

The pyrimidine side product, **2.10**, had the following spectroscopic characteristics:[87]



^{1}H NMR (500 MHz, $CDCl_3$): δ 2.57 (s, 3H), 6.96 (t, J = 4.8 Hz, 1H), 8.52 (d, J = 4.8 Hz, 2H).

Methyl 2-chloronicotinoate 2.12

To a stirred solution of acid chloride **2.3** (965 mg, 5.5 mmol) and TEA (1.14 mL, 8.2 mmol) in CH_2Cl_2 (10 mL) at 0 °C was added dry MeOH (2.22 mL, 55 mmol) dropwise over 3 min. A precipitate intitially formed during this addition but redissolved before the addition was complete. The solution was stirred for a further 30 min at rt then concentrated *in vacuo*. The light brown residues thus obtained were dissolved in CH_2Cl_2 (25 mL), washed with sat aq $NaHCO_3$ (25 mL), sat brine solution (25 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo* to afford the methyl ester as a light brown oil (798 mg, 85% yield).

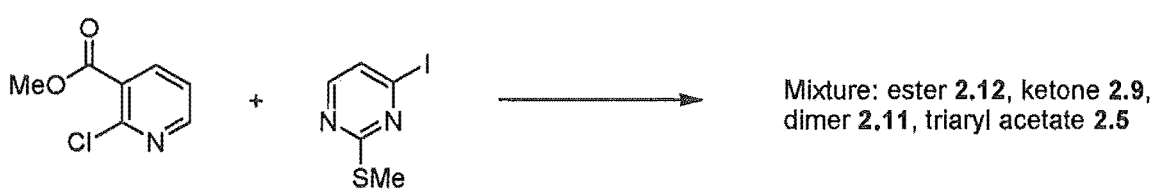
^{1}H NMR (500 MHz, $CDCl_3$): δ 3.97 (s, 3H, H-9), 7.36 (dd, J = 4.8, 7.6 Hz, 1H, H-5), 8.19 (dd, J = 1.9, 7.6 Hz, 1H, H-4), 8.52 (dd, J = 1.9, 4.8 Hz, 1H, H-6).

^{13}C NMR (75 MHz, $CDCl_3$): δ 52.7 (C-9), 122.0 (C-5), 126.5 (C-3), 140.1 (C-4), 149.7 (C-6), 151.7 (C-2), 164.6 (C-7).

IR (CH_2Cl_2 soln): υ_{max} 1740, 1582, 1562, 1450, 1435, 1406, 1308, 1265, 1242, 1194, 1144, 1067, 961, 835, 820, 762-683 (series of sharp bands), 646.

HRMS (ESI): Calcd for $C_7H_7{}^{35}ClNO_2$ (MH^+) *m/z* = 172.0165, found *m/z* = 172.0167.

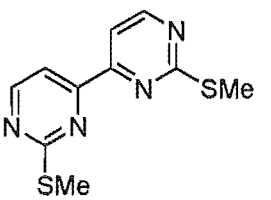
Unsuccessful Grignard arylation of 2.12



To a vigorously stirred solution of iodide **2.1** (441 mg, 1.75 mmol) in toluene (11 mL) at 0 °C was added a solution of *i*-PrMgCl in diethyl ether (1.50 M, 1.17 mL, 1.75 mmol) dropwise over 5 min, and the resulting light brown solution stirred at 0 °C for 1.5 h. Into this was transferred,

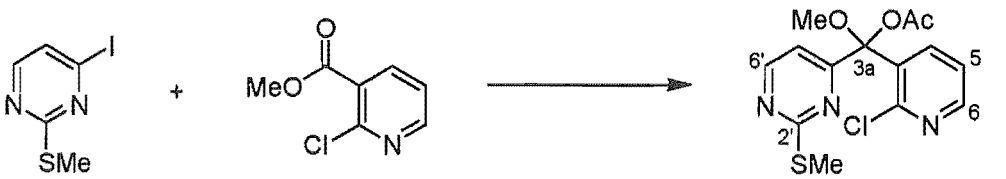
via cannula, a similarly cooled solution of methyl ester **2.12** (100 mg, 0.58 mmol) in toluene (8 mL) dropwise over 5 min, and the resulting brown solution was stirred at 0 °C for 4 h. To this was added sat aq $NaHCO_3$ (20 mL) and the biphasic solution extracted with EtOAc (3 × 10 mL). The organic extractions were combined, dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was examined by ^1H NMR and shown to contain a mixture of compounds, including triaryl acetate **2.5**, ketone **2.9**, pyrimidine **2.10** and bipyrimidine **2.11**. Purification of the mixture was not carried out.

Bipyrimidine side product **2.11** had the following spectroscopic characteristics:[87]



^1H NMR (500 MHz, $CDCl_3$): δ 2.65 (s, 6H), 8.04 (d, J = 5.1 Hz, 2H), 8.71 (d, J = 5.1 Hz, 2H).

Acetic acid (2-chloro-pyridin-3-yl)-methoxy-(2-methylsulfanyl-pyrimidin-4-yl)-methyl ester 2.13



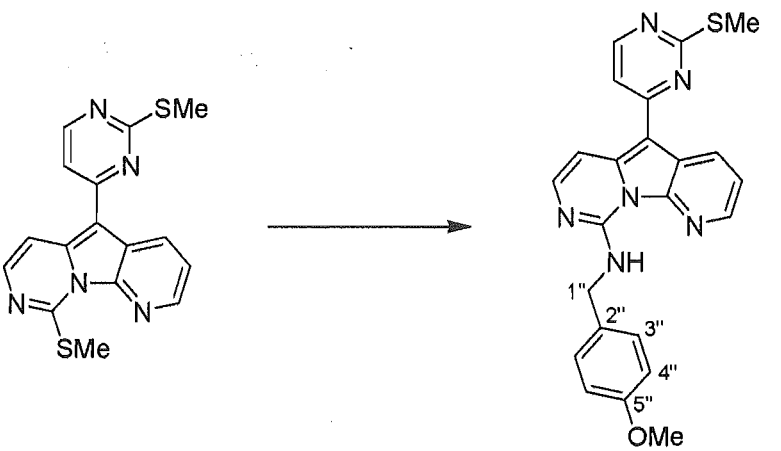
To a vigorously stirred solution of iodide **2.1** (700 mg, 2.78 mmol) and methyl ester **2.12** (159 mg, 0.93 mmol) in THF (9 mL) at -78°C was added a solution of *n*-BuLi in hexanes (1.52 M, 1.83 mL, 2.78 mmol) dropwise over 5 min. The dark brown solution was stirred at -78 °C for 30 min, then AcCl (330 μL, 4.63 mmol) was added dropwise over 5 min. The deep red solution was stirred at rt for 3 h, then diluted with CH_2Cl_2 (20 mL), washed with sat aq $NaHCO_3$ (20 mL), sat brine solution (20 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by flash chromatography on silica, eluting with 1:1 EtOAc/hexanes, to afford the quaternary product as an orange/brown amorphous solid (293 mg, 93% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.23 (s, 3H, $COOCH_3$), 2.34 (s, 3H, SCH_3), 3.32 (s, 3H, OCH_3), 7.37 (dd, J = 4.8, 7.9 Hz, 1H, H-5), 7.65 (d, J = 5.2 Hz, 1H, H-5'), 8.37 (dd, J = 2.0, 4.8 Hz, 1H, H-6), 8.46 (dd, J = 2.0, 7.9 Hz, 1H, H-4), 8.60 (d, J = 5.2 Hz, 1H, H-6').

^{13}C NMR (75 MHz, $CDCl_3$): δ 13.4 (SCH_3), 20.7 ($COOCH_3$), 50.6 (OCH_3), 100.3 (C-3a), 114.7 (C-5'), 121.9 (C-5), 132.7 (C-3), 139.0 (C-4), 146.9 (C-2), 149.2 (C-6), 156.8 (C-6'), 164.5 (C-4'), 166.6 ($COOCH_3$), 171.7 (C-2').

LRMS (ESI): Calcd for $C_{14}H_{15}{}^{35}ClN_3O_3{}^{32}S$ (MH^+) *m/z* = 340.05, found *m/z* = 340.08.

9-(4-Methoxybenzylamino)-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.17



Variolin core structure **2.6** (100 mg, 0.29 mmol) was heated with PMB (190 μL, 1.47 mmol) in toluene (2.0 mL) at 100 °C for 3 d. The solution was allowed to cool to rt then concentrated *in vacuo*. The crude material thus obtained was subjected to flash chromatography on silica, eluting with 70% EtOAc/hexanes, to give the monosubstituted product as an orange foam (124 mg, 99 % yield).

Mp: 66-68 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.67 (s, 3H, 2'-SCH_3), 3.81 (s, 3H, OCH_3), 4.88 (d, J = 5.4, 2H, H-1"), 6.92 (d, J = 8.8 Hz, 2H, H-4"), 7.26 (d, J = 5.4 Hz, 1H, H-5'), 7.40 (dd, J = 4.4, 7.8 Hz, 1H, H-3), 7.41 (d, J = 8.7 2H, H-3"), 7.47 (d, J = 6.8 Hz, 1H, H-6), 7.72 (d, J = 6.3 Hz, 1H, H-7), 8.27 (dd, J = 1.0, 3.4 Hz, 1H, H-2), 8.44 (d, J = 5.4 Hz, 1H, H-6'), 8.65 (dd, J = 1.0, 6.8 Hz, 1H, H-4), 10.38 (t, J = 5.4 Hz, 1H, 9-N*H*).

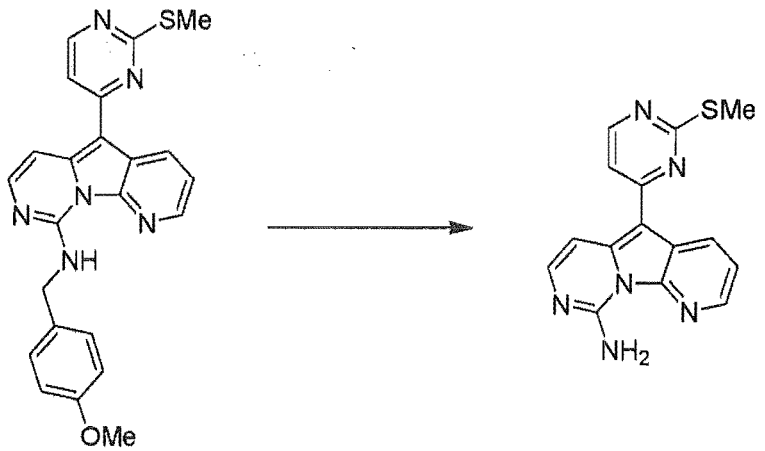
^{13}C NMR (75 MHz, $CDCl_3$): δ 14.3 (2'-SCH_3), 44.4 (C-1''), 55.3 (OCH_3), 99.7 (C-5), 101.3 (C-6), 112.4 (C-5'), 114.1 (PMB C-4''), 120.3 (C-3), 121.7 (C-4a), 128.3 (C-4), 128.9 (C-3''), 130.1 (C-2''), 139.1 (C-5a), 139.8 (C-2), 143.5 (C-10a), 144.0 (C-7), 149.1 (C-9), 156.3 (C-6'), 159.0 (C-5''), 161.3 (C-4'), 172.2 (C-2').

IR (KBr): υ_{max} 3060-2850 (series of weak bands), 1614, 1558, 1475, 1364, 1248, 1182, 1032, 806.

HRMS (ESI): Calcd for $C_{23}H_{21}N_6O^{32}S$ (MH^+) *m/z* = 429.1498, found *m/z* = 429.1492.

IC_{50} (P388): >29 μM

9-Amino-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine



PMB-protected variolin **2.17** (124 mg, 0.29 mmol) was dissolved in triflic acid (3.0 mL), and the deep red/orange solution stirred for 2 h at rt. The solution was then cooled to 0 °C and aq NH_3 solution added dropwise until a yellow precipitate had formed and the solution was alkaline. Sat aq $NaHCO_3$ solution was slowly added with stirring, and the resulting yellow suspension extracted with CH_2Cl_2 (50 mL). The yellow organic solution was concentrated *in vacuo*, and the crude material thus obtained purified by flash chromatography on neutralised silica, eluting with 2% MeOH/CH_2Cl_2 (0.1% TEA), to give the deprotected product as a yellow microcrystalline solid (84 mg, 94% yield).

Mp: dec 237-242 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.69 (s, 3H, SCH_3), 5.8-6.4 (br s, 1H, N*H*a), 7.33 (d, J = 5.5 Hz, 1H, H-5'), 7.49 (dd, J = 4.8, 8.3 Hz, 1H, H-3), 7.58 (d, J = 6.7 Hz, 1H, H-7), 7.68 (d, J = 6.3 Hz,

1H, H-6), 8.40 (dd, J = 1.6, 4.8 Hz, 1H, H-2), 8.49 (d, J = 5.2 Hz, 1H, H-6'), 8.74 (dd, J = 1.6, 8.3 Hz, 1H, H-4), 9.4-10.2 (br s, 1H, N*H*b).

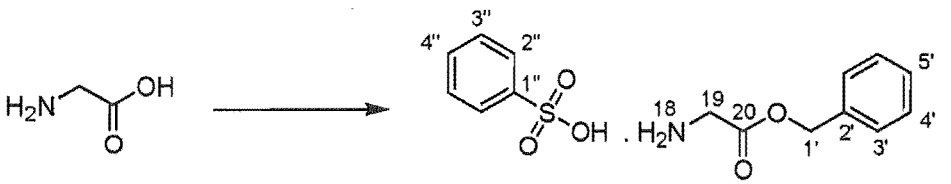
^{13}C NMR (75 MHz, $CDCl_3$): δ 14 .3 (2'-SCH_3), 100.2 (C-5), 102.2 (C-6), 112.6 (C-5'), 120.7 (C-3), 122.0 (C-4a), 128.6 (C-4), 138.8 (C-5a), 140.3 (C-2), 143.4 (C-10a), 143.7 (C-7), 149.8 (C-9), 156.5 (C-6'), 161.4 (C-4'), 172.4 (C-2').

IR (KBr): υ_{max} 3321, 3117 (broad), 3016 (broad), 2341, 2341, 1917, 1647, 1558, 1516, 1454, 1404, 1323, 1269, 1196, 1010, 980, 818.

HRMS (ESI): Calcd for $C_{15}H_{13}N_6{}^{32}S$ (MH^+) *m/z* = 309.0922, found *m/z* = 309.0921.

IC_{50} (P388): 3.9 μM

Glycine benzyl ester benzenesulfonate[138]

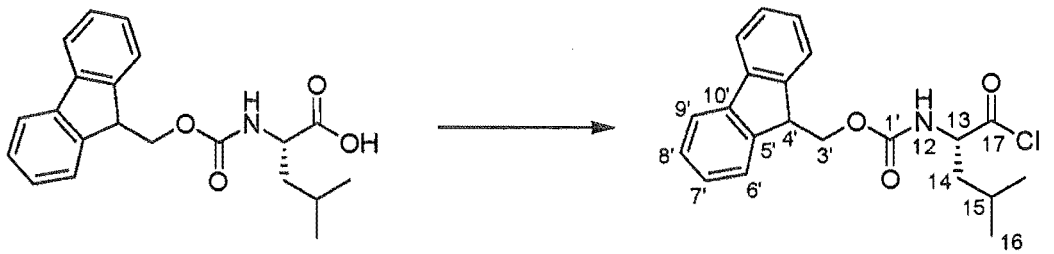


A solution of glycine (1.00 g, 13.3 mmol) and benzenesulfonic acid (2.32 g, 14.6 mmol) in benzyl alcohol (8.00 mL, 77.3 mmol) was heated to 130 °C under reduced pressure (9 mm Hg) for 2 h, then kept at 100 °C under reduced pressure (0.03 mm Hg) for 1 h. During this time a small amount of benzyl alcohol was collected by distillation (~1 mL). The thick oil was poured into a mortar and pestle, allowed to cool to rt, then ground with diethyl ether (20 mL) to give the ester salt as a white crystalline solid. This solid was collected by filtration, washed with diethyl ether, and dried *in vacuo* (3.71 g, 86% yield).

^{1}H NMR (500 MHz, CD_3OD): δ 3.87 (s, 2H, H-19), 5.25 (s, 2H, H-1'), 7.31-7.45 (m, 8H, H-3', H-3", H-4', H-4", H-5'), 7.82 (dd, J = 1.7, 7.6 Hz, 2H, H-2').

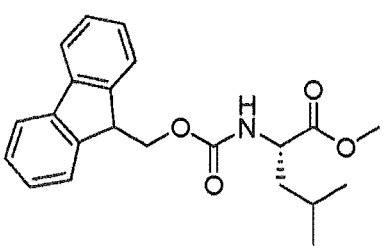
^{13}C NMR (75 MHz, CD_3OD): δ 41.4 (C-19), 69.2 (C-1'), 127.2 (C-3"), 129.6 (Ar), 129.92 (Ar), 129.93 (Ar), 129.96 (Ar), 131.6 (C-2" or C-4"), 136.7 (C-2'), 168.7 (C-20).

N-(9-Fluorenylmethoxycarbonyl)-L-leucine acid chloride



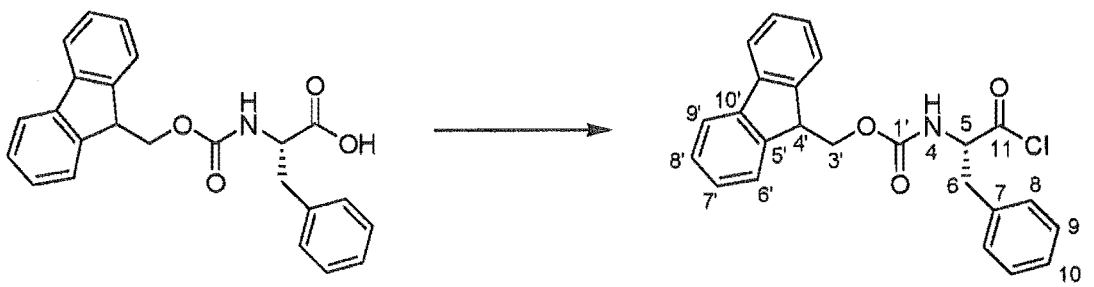
This was prepared from Fmoc-L-leucine, via the slightly modified procedure of Carpino *et al.*[95] A solution of Fmoc-L-leucine (500 mg, 1.41 mmol) in a mixture of CH_2Cl_2 (2.5 mL), thionyl chloride (2.0 mL) and DMF (10 μL) was heated to reflux for 1.5 h. The solution was allowed to cool to rt then concentrated *in vacuo*. The residues thus obtained were dissolved in benzene (5 mL) and concentrated *in vacuo*; this process of dissolution and concentration was repeated twice more. The residues thus obtained were left at reduced pressure (0.03 mm Hg) at 45 °C for 48 h to give the acid chloride as a white amorphous solid (523 mg, 99% yield). Examination of this material by ^{1}H NMR was hindered by hydrolysis of the acid chloride by traces of water in the $CDCl_3$ NMR solvent, so that mixtures of the acid chloride and the acid were invariably observed. Therefore, in order to confirm the purity of the acid chloride sample, a small amount was converted to the methyl ester as follows.

To a small amount of Fmoc-L-leucine acid chloride was added dry MeOH and the resulting solution stirred at rt for 10 min then concentrated *in vacuo*. The methyl ester thus obtained was shown by ^{1}H NMR spectroscopy to be free from contamination by the acid, and had the following spectroscopic properties:



^{1}H NMR (500 MHz, $CDCl_3$): δ 0.947 (d, J = 3.4 Hz, 3H, H-16a), 0.960 (d, J = 3.4 Hz, 3H, H-16b), 1.50-1.75 (m, 3H, H-24 and H-15), 3.74 (s, 3H, OMe), 4.23 (t, J = 7.0 Hz, 1H, H-4'), 4.42 (d, J = 7.3, 2H, H-3'), 5.20 (br, 1H, H-12), 7.32 (t, J = 7.6 Hz, 2H, H-7'), 7.40 (t, J = 7.6 Hz, 2H, H-8'), 7.60 (t, J = 6.6 Hz, 2H, H-6'), 7.76 (d, J = 7.3 Hz, 2H, H-9').

_N_-(9-Fluorenylmethoxycarbonyl)-L-phenylalanine acid chloride



This was prepared from Fmoc-L-phenylalanine via the slightly modified procedure of Carpino *et al.*[95] A solution of Fmoc-L-phenylalanine (2.00 g) in a mixture of CH_2Cl_2 (10 mL) and thionyl chloride (4 mL) was heated to reflux for 20 min. THF (2 mL) was added and the solution heated to reflux for a further 30 min. The solution was allowed to cool then concentrated *in vacuo* to give a colourless oil. The oily residues were dissolved in CH_2Cl_2 (10 mL), concentrated *in vacuo*, then again dissolved in CH_2Cl_2 (10 mL) and concentrated *in vacuo*. The residues thus obtained were dissolved in CH_2Cl_2 (10 mL) and diluted, with vigorous stirring, with hexanes (100 mL). The resulting precipitate was collected by filtration and dried *in vacuo* to afford the protected amino acid chloride as white needles (1.91 g, 91% yield).

Mp: 114-115 °C (lit. 120-1 °C).[95]

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.22 (dd, J = 6.8, 14.2 Hz, 1H, H-6a), 3.27 (dd, J = 5.4, 14.7 Hz, 1H, H-6b), 4.20 (t, J = 6.8 Hz, 1H, H-4'), 4.42 (d, J = 6.8, 2H, H-3'), 4.88 (dt, J = 6.3, 8.3 1H, H-5), 5.14 (d, J = 7.8 Hz, 1H, H-4), 7.17 (d, J = 6.8 Hz, 2H, H-6'), 7.28-7.37 (m, 5H, H-8, H-9 and H-10), 7.41 (t, J = 7.3 Hz, 2H, H-7'), 7.52 (t, J = 7.3 Hz, 2H, 8'), 7.77 (d, J = 7.3 Hz, 2H, H-9').

_N_-(9-Fluorenylmethoxycarbonyl)glycine acid chloride

This was prepared from Fmoc-glycine via the slightly modified procedure of Carpino *et al.*[95] A solution of Fmoc-glycine (500 mg, 1.68 mmol) in a mixture of CH_2Cl_2 (2.0 mL), THF (0.5 mL) and thionyl chloride (1.0 mL) was heated to reflux for 30 min. The solution was allowed to cool then concentrated *in vacuo*. The residues were dissolved in CH_2Cl_2 (5 mL) and the solution concentrated *in vacuo*; this process of dissolution and concentrated was repeated. The residues thus obtained were dissolved in CH_2Cl_2 (5 mL) and diluted, with vigorous stirring, with hexanes (25 mL). The resulting precipitate was collected by filtration and dried *in vacuo* to afford the protected amino acid chloride as a white powder (465 mg, 88% yield).

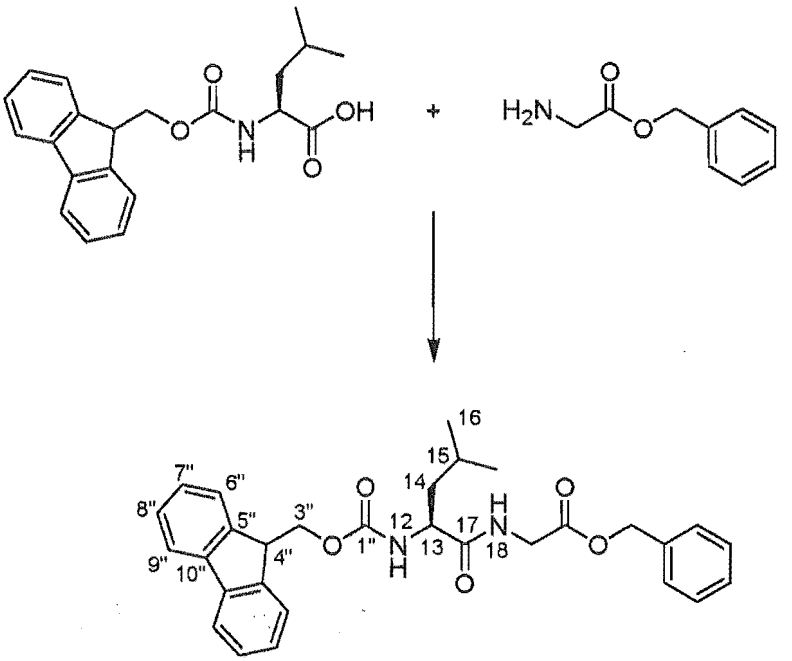
Mp: 107-108 °C (lit. 108-9°C).[95]

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.24 (t, J = 6.8 Hz, 1H, H-4'), 4.38 (d, J = 6.3, 2H, H-3'), 4.47 (d, J = 6.8 Hz, 2H, H-2), 5.32 (br, 1H, H-1), 7.31 (t, J = 7.1 Hz, 2H, H-7'), 7.41 (t, J = 7.6 Hz, 2H, H-8'), 7.58 (d, J = 7.3 Hz, 2H, H-6'), 7.77 (d, J = 7.3 Hz, 2H, H-9').

Phosphate buffer, pH = 5.5[98]

$NaH_2PO_4.2H_2O$ (94.2 g, 529 mmol) and Na_2HPO_4 (30.4 g, 253 mmol) were dissolved in H_2O (1.5 L), and a small amount of dil aq H_3PO_4 was added to lower the pH from 5.7 to 5.5.

N-(9-Fluorenylmethoxycarbonyl)-L-leucyl-glycine benzyl ester



To a solution of Fmoc-L-leucine (1.95 g, 5.5 mmol), glycine benzyl ester benzenesulfonate (1.78 g, 5.5 mmol), HOBT (1.12 g, 8.3 mmol), and TEA (845 μL, 6.1 mmol) in THF (150 mL) was added DCC (1.48 g, 7.2 mmol) and the solution left at 4 °C for 18 h. The resulting fine suspension was concentrated *in vacuo* to leave a white solid, which was purified by flash chromatography on silica, eluting with 15% EtOAc/CH_2Cl_2, to afford the dipeptide as a white amorphous solid (2.80 g, 90% yield).

Mp: 106-108 °C.

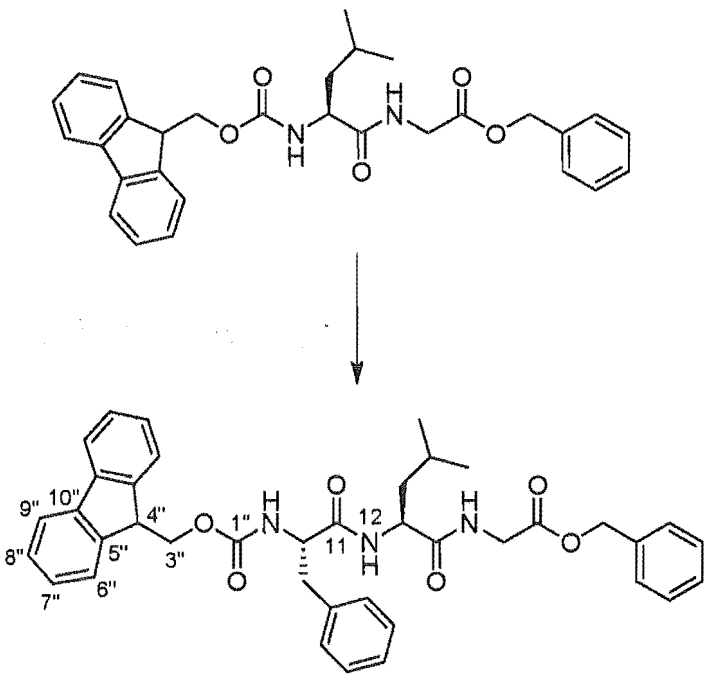
^{1}H NMR (500 MHz, d_6-DMSO): δ 0.84 (d, J = 6.3 Hz, 3H, H-16a), 0.87 (d, J = 6.3 Hz, 3H, H-16b), 1.41-1.54 (m, 2H, H-14), 1.60-1.69 (m, 1H, H-15), 3.86 (dd, J = 5.4 17.6 Hz, 1H, H-19a), 3.96 (dd, J = 5.9, 17.1 Hz, 1H, H-19b), 4.10 (td, J = 4.9, 9.0, 1H, H-13), 4.22 (m, 2H, H-3"), 4.31 (m, 1H, H-4"), 5.11 (s, 2H, H-1'), 7.29-7.37 (m, 7H, H-3', H-4', H-5', H-7"), 7.41 (t, J = 7.3 Hz, 2H, H-8"), 7.55 (d, J = 8.8 Hz, 1H, H-12), 7.73 (t, J = 6.1 Hz, 2H, H-6"), 7.88 (d, J = 7.3 Hz, 2H, H-9"), 8.40 (t, J = 5.9 Hz, 1H, H-18).

^{13}C NMR (75 MHz, d_6-DMSO): δ 21.5 (C-16a), 23.2 (C-16b), 24.3 (C-15), 40.87 (C-14), 40.92 (C-19), 46.9 (C-13), 65.7 (C-3"), 66.0 (C-1'), 120.2 (C-9"), 125.5 (C-6"), 127.2 (C-7"), 127.8 (C-8"), 128.08 (Ar), 128.21 (Ar), 128.5 (Ar), 136.0 (C-2'), 140.9 (C-10"), 143.9 (C-5"), 156.1 (C-1"), 169.8 (C-20), 173.2 (C-17).

IR (KBr): υ_{max} 3288, 3065, 2941, 1734, 1695, 1653, 1539, 1452, 1239, 1119, 1034, 980, 739, 698.

HRMS (ESI): Calcd for $C_{30}H_{33}N_2O_5$ (MH^+) *m/z* = 501.2389, found *m/z* = 501.2396.

***N*-(9-Fluorenylmethoxycarbonyl)-L-phenylalanyl-L-leucylglycine benzyl ester**



A solution of FmocLGOBn (1.98g, 3.95 mmol) in 1:1 $CHCl_3$/AMP (90 mL) was stirred at rt for 5 min. The solution was diluted with $CHCl_3$ (350 mL) and washed with sat brine solution (2 × 400 mL), then pH=5.5 phosphate buffer (2 × 400 mL). To the organic solution was immediately added Fmoc-L-phenylalanine acid chloride (1.96 g, 4.82 mmol) and 5% aq Na_2CO_3 (35 mL), and the biphasic solution stirred vigorously at rt for 45 min. The organic layer was isolated and washed with water (300 mL), sat brine solution (300 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo* to leave an off-white oily residue. To the crude material thus obtained was added CH_2Cl_2 (250 mL) and silica (10 g), and the resulting slurry sonicated with swirling for 5 min then loaded onto a column of silica (90 g) in CH_2Cl_2. Flash chromatography, eluting with 20% EtOAc/CH_2Cl_2, afforded the tripeptide as a white amorphous solid (1.63 g, 64% yield).

Mp: 160-162 °C.

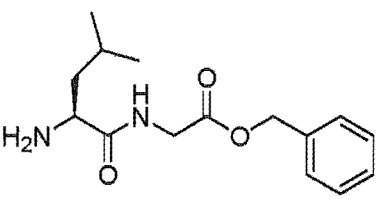
^{1}H NMR (500 MHz, d_6-DMSO): δ 0.81 (d, J = 6.2 Hz, 3H, H-9a), 0.84 (d, J = 6.7 Hz, 3H, H-9b), 1.46 (t, J = 7.2 Hz, 2H, H-14), 1.62 (p, J = 6.7 Hz, 1H, H-15), 2.77 (dd, J = 10.9, 13.4 Hz, 1H, H-6a), 3.02 (dd, J = 3.6, 13.4 Hz, 1H, H-6b), 3.86 (dd, J = 5.7, 17.6 Hz, 1H, H-19a), 3.95 (dd, J = 5.7, 17.6 Hz, 1H, H-19b), 4.11-4.17 (m, 3H, H-3" and H-4"), 4.30 (td, J = 3.6, 9.6 Hz, 1H, H-5), 4.38 (q, J = 7.8 Hz, 1H, H-13), 5.11 (s, 2H, H-1'), 7.17 (t, J = 7.2 Hz, 1H, H-10), 7.23-7.37 (m, 11H, H-3', H-4', H-5', H-7", H-8 and H-9), 7.40 (td, J = 2.6, 7.5 Hz, 2H, H-8"), 7.57-7.64 (m, 3H, H-6" and H-4), 7.87 (d, J = 7.8 Hz, 2H, H-9"), 8.08 (d, J = 8.3 Hz, 1H, H-12), 8.37 (t, J = 5.9 Hz, 1H, H-18).

^{13}C NMR (75 MHz, d_6-DMSO): δ 21.8 (C-16a), 23.1 (C-16b), 24.2 (C-15), 37.5 (C-6), 40.9 (C-19), 41.3 (C-14), 46.7 (C-4"), 51.0 (C-13), 56.1 (C-5), 65.8 (C-3"), 66.1 (C-1'), 120.2 (C-9"), 125.4 (C-6"), 126.6 (C-10), 127.2 (C-7"), 127.8 (C-8"), 128.1 (C-9), 128.17 (OBn), 128.24 (OBn), 128.6 (OBn), 129.4 (C-8), 136.0 (C-2'), 138.3 (C-7), 140.8 (C-10"), 143.9 (C-5"), 155.9 (C-1'), 169.8 (C-20), 171.5 (C-11), 172.7 (C-17).

IR (KBr): υ_{max} 3279, 3065, 2948, 1690, 1647, 1545, 1452, 1261, 1196, 1143, 1086, 1040, 993, 741, 698.

HRMS (ESI): Calcd for $C_{39}H_{42}N_3O_6$ (MH^+) *m/z* = 648.3074, found *m/z* = 648.3082.

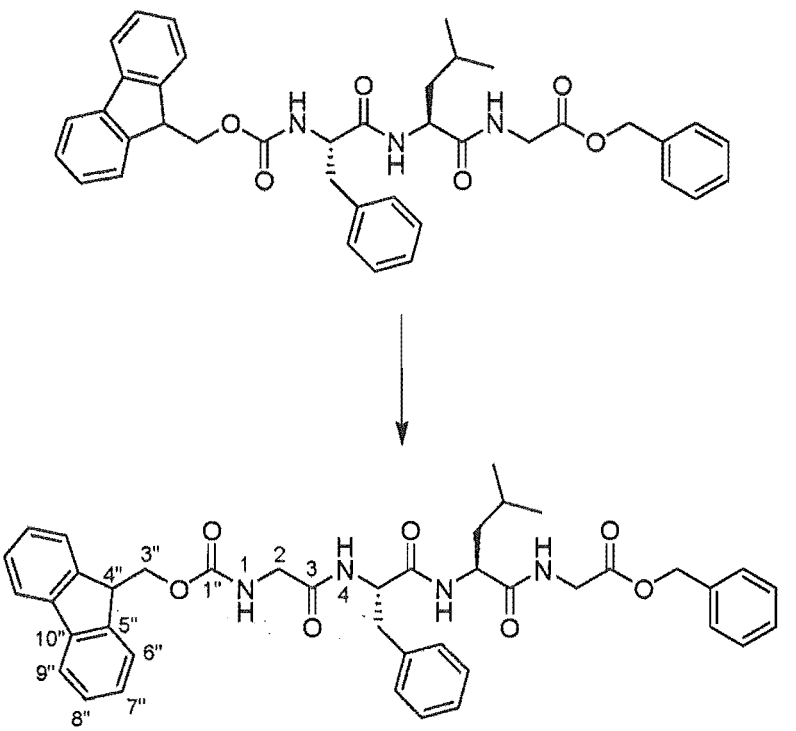
The deprotected dipeptide intermediate, LGOBn, had the following spectroscopic characteristics:



^{1}H NMR (500 MHz, d_6-DMSO): δ 0.82 (d, J = 6.3, 3H, H-16a), 0.86 (d, J = 6.3 Hz, 3H, H-16b), 1.21 (ddd, J = 5.9, 8.8, 13.2 Hz, 1H, H-14a), 1.39 (ddd, J = 5.4, 6.3, 13.2 Hz, 1H, H-14b), 1.72 (pd, J = 2.4, 5.9 Hz, 1H, H-15), 3.18 (dd, J = 5.4, 9.3 Hz, 1H, H-13), 3.87 (dd, J = 5.4, 17.6 Hz, 1H, H-19a), 3.92 (dd, J = 5.4, 17.6 Hz, 1H, H-19b), 4.49 (s, 2H, H-12), 5.11 (s, 2H, H-1'), 7.29-7.37 (m, 5H, H-3', H-4' and H-5'), 8.33 (t, J = 5.4 Hz, 1H, H-18).

^{13}C NMR (75 MHz, d_6-DMSO): δ 21.9 (C-16a), 23.3 (C-16a), 24.1 (C-15), 40.8 (C-19), 44.4 (C-14), 53.0 (C-13), 66.0 (C-1'), 128.10 (Ar), 128.23 (Ar), 128.57 (Ar), 136.0 (C-2'), 170.0 (C-20), 176.5 (C-17).

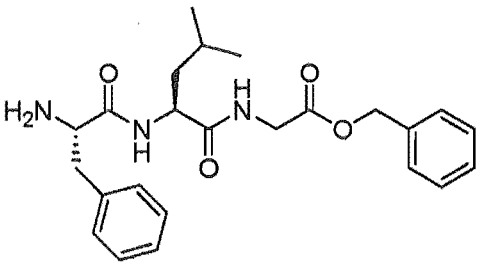
N-(9-Fluorenylmethoxycarbonyl)glycyl-L-phenylalanyl-L-leucylglycine benzyl ester



a) From FmocFLGOBn:

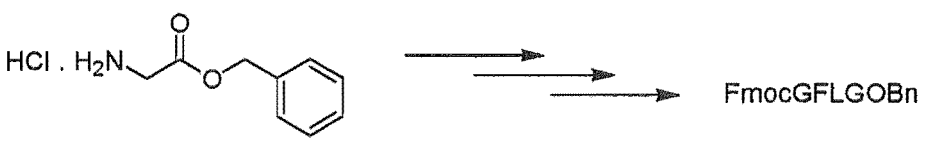
A solution of FmocFLGOBn (1.23 g, 1.9 mmol) in 6:1 $CHCl_3$/AMP (70 mL) was stirred at rt for 1.5 h. The solution was diluted with $CHCl_3$ (100 mL), washed with sat brine solution (2 × 100 mL) and pH = 5.5 phosphate buffer (2 × 100 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. To the oily residues thus obtained were added EDCI (473 mg, 2.47 mmol), HOBT (385 mg, 2.85 mmol), Fmoc-glycine (437 mg, 1.47 mmol), THF (120 mL) and TEA (342 μL, 2.47 mmol) and the resulting white suspension stirred at rt for 16 h. The suspension was washed with 1:3 1.0 M aq HCl/sat brine solutions (200 mL), diluted with THF (60 mL), washed with sat brine solution (200 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by repeated flash chromatography on silica, eluting with 2:1 CH_2Cl_2/EtOAc (2% MeOH), to give the tetrapeptide as a white amorphous solid (1.11 g, 83% yield).

The deprotected tripeptide intermediate, FLGOBn, had the following spectroscopic characteristics:



^{1}H NMR (500 MHz, d_6-DMSO): δ 0.82 (d, J = 6.3 Hz, 3H, H-16a), 0.84 (d, J = 6.3 Hz, 3H, H-16b), 1.40-1.63 (m, 3H, H-14 and H-15), 2.64 (dd, J = 8.3, 13.7 Hz, 1H, H-6a), 2.95 (dd, J = 4.4, 13.2 Hz, 1H, H-6b), 3.32 (br s, 2H, H-4), 3.46 (dd, J = 4.4, 8.3 Hz, 1H, H-5), 3.84 (dd, J = 5.9, 17.6 Hz, 1H, H-19a), 3.92 (dd, J = 5.9, 17.1 Hz, 1H, H-19b), 4.36 (p, J = 6.8 Hz, 1H, H-13), 5.11 (s, 2H, H-1'), 7.16-7.28 (m, 5H, H-8, H-9 and H-10), 7.34-7.38 (m, 5H, H-3', H-4' and H-5'), 8.00 (d, J = 8.3 Hz, 1H, H-12), 8.41 (t, J = 5.9 Hz, 1H, H-18).

b) Continuous solution phase synthesis with Fmoc-amino acid chlorides:



FmocGFLGOBn was synthesised via the slightly modified procedure of Carpino *et al.*[95,98] To a suspension of glycine benzyl ester hydrochloride salt (70 mg, 0.22 mmol) in $CHCl_3$ (2 mL) was added a solution of Fmoc-L-leucine acid chloride (105 mg, 0.28 mmol) in $CHCl_3$ (2 mL), followed immediately by 5% aq Na_2CO_3 (2 mL). The biphasic solution was stirred vigorously for 10 min, transferred to a separating funnel, a small amount of sat brine solution added to assist phase separation, and the organic layer collected. AMP (2.5 mL) was added and the resulting solution stirred at rt for 30 min, diluted with $CHCl_3$ (15 mL) and extracted with pH = 5.5 phosphate buffer (5 × 10 mL). The organic solution was concentrated *in vacuo* to a volume of ~4 mL, and the coupling reaction repeated as above, but with Fmoc-L-phenylalanine acid chloride (114 mg, 0.28 mmol) as the electrophile. The Fmoc deprotection manipulations were carried out exactly as before to give a solution of the deprotected tripeptide in $CHCl_3$ (~4 mL). A final coupling round was carried out as above, but with Fmoc-glycine acid chloride (89 mg, 0.28 mmol) as the electrophile. Following isolation of the organic layer as before, 1,2-diaminoethane (50 μL, 0.75 mmol) was added, the solution stirred for 1 min, extracted with pH = 5.5 phosphate buffer (2 × 20 mL) and sat brine solution (20 mL), dried with Na_2SO_4,

filtered and concentrated *in vacuo*. The oily residues thus obtained were dissolved in DMSO (5 mL) with sonicating to give a milky solution. CH_2Cl_2 (40 mL) was added (with a concomitant increase in the amount of the fine precipitate, which was isolated and shown by NMR spectroscopy to be non-peptidic) and the resulting milky solution extracted with H_2O (3 × 50 mL) and sat brine solution (50 mL) (the solution cleared with the sat brine solution wash), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by flash chromatography on neutralised silica, eluting with 2:1 CH_2Cl_2/EtOAc (2.5% MeOH), to give the title compound as a white amorphous solid (45 mg, 95% pure, 28% yield).

Mp: 77-80 °C.

^{1}H NMR (500 MHz, d_6-DMSO): δ 0.80 (d, J = 6.3 Hz, 3H, H-16a), 0.85 (d, J = 6.8 Hz, 3H, H-16b), 1.45 (t, J = 7.1 Hz, 2H, H-14), 1.59 (p, J = 6.8 Hz, 1H, H-15), 2.76 (dd, J = 9.3, 14.1 Hz, 1H, H-6a), 3.01 (dd, J = 4.4, 13.7 Hz, 1H, H-6b), 3.50 (dd, J = 5.9, 16.6 Hz, 1H, H-2a), 3.63 (dd, J = 5.9, 16.6 Hz, 1H, H-2b), 3.83 (dd, J = 5.9, 17.6 Hz, 1H, H-19a), 3.92 (dd, J = 5.9, 17.1 Hz, 1H, H-19b), 4.20 (t, J = 6.8 Hz, 1H, H-4"), 4.24 (d, J = 6.3 Hz, 2H, H-3"), 4.32 (q, J = 7.8 Hz, 1H, H-13), 4.54 (td, J = 4.4, 8.8 Hz, 1H, H-5), 5.11 (s, 2H, H-1'), 7.13-7.17 (m, 1H, H-10), 7.19-7.22 (m, 4H, H-8 and H-9), 7.30-7.37 (m, 7H, H-3', H-4', H-5' and H-7"), 7.41 (t, J = 7.3 Hz, 2H, H-8"), 7.51 (t, J = 6.3 Hz, 1H, H-1), 7.69 (d, J = 7.8 Hz, 2H, H-6"), 7.88 (d, J = 7.3 Hz, 2H, H-9"), 8.00 (d, J = 8.3 Hz, 1H, H-4), 8.11 (d, J = 8.3 Hz, 1H, H-12), 8.27 (t, J = 5.9 Hz, 1H, H-18).

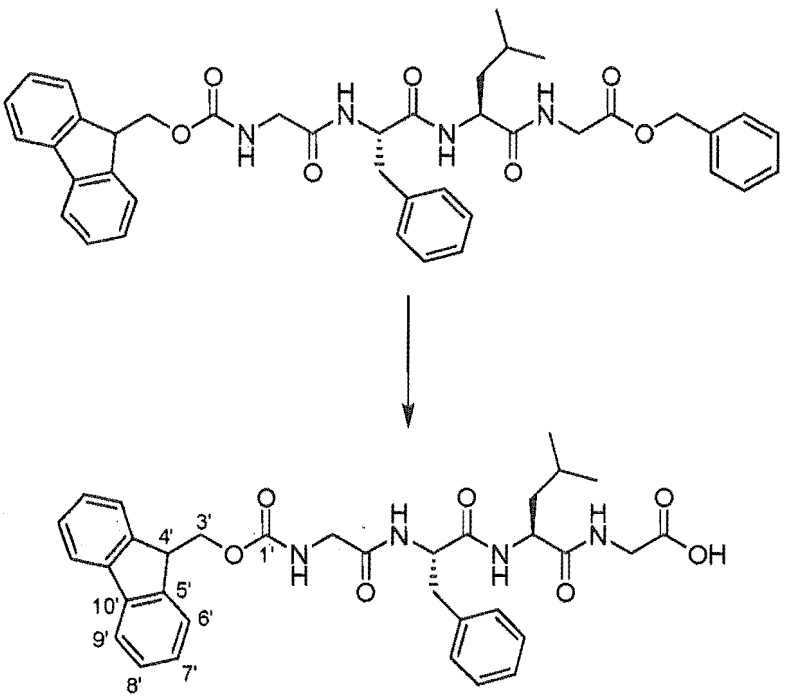
^{13}C NMR (75 MHz, d_6-DMSO): δ 21.8 (C-16a), 23.1 (C-16b), 24.2 (C-15), 37.7 (C-6), 40.9 (C-19), 41.1 (C-14), 43.5 (C-2), 46.8 (C-4"), 51.1 (C-13), 53.9 (C-5), 65.99 (C-3"), 66.07 (C-1'), 120.3 (C-9"), 125.4 (C-6"), 126.4 (C-10), 127.3 (C-7"), 127.82 (C-8"), 128.13 (C-9), 128.18 (Ar), 128.25 (Ar), 128.58 (Ar), 129.4 (C-8), 136.0 (C-2'), 137.8 (C-7), 140.9 (C-10"), 144.0 (C-5"), 156.7 (C-1"), 169.2 (C-3), 169.7 (C-20), 171.0 (C-11), 172.7 (C-17).

IR (KBr): υ_{max} 3275, 3055, 2955, 1653, 1522, 1452, 1399, 1356, 1257, 1192, 1107, 1043, 995, 743, 698.

HRMS (ESI): Calcd for $C_{41}H_{45}N_4O_7$ (MH^+) *m/z* = 705.3288, found *m/z* = 705.3284.

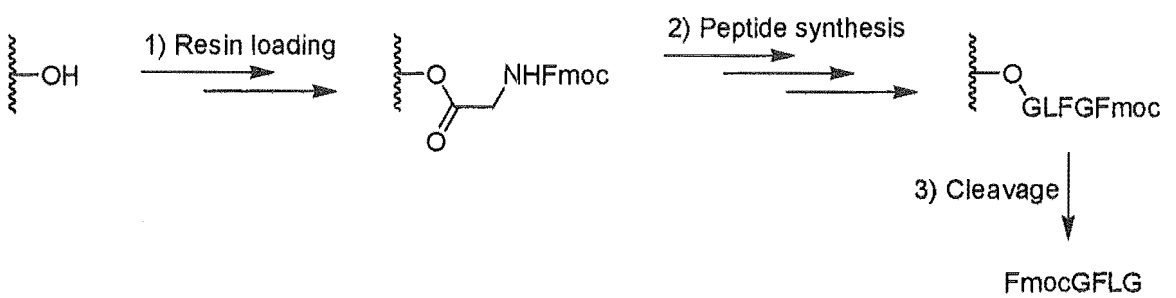
N-(9-Fluorenylmethoxycarbonyl)glycyl-L-phenylalanyl-L-leucylglycine

a) From FmocGFLGOBn:



To a solution of FmocGFLGOBn (700 mg, 0.99 mmol) in THF (60 mL) was added 10% palladium on charcoal (700 mg). The black suspension was subjected to 3 vacuum/H_2 cycles, then stirred under H_2 for 16 h at rt. The suspension was passed through a celite plug and the colourless filtrate concentrated *in vacuo* to a volume of around 25 mL. To this was added hexanes (200 mL) with vigorous stirring, and the resulting gel-like precipitate collected by filtration to afford the deprotected tetrapeptide as a white amorphous solid (496 mg, 98% pure, 80% yield).

b) Solid phase synthesis:[94]



1) Loading: Wang resin (1.00 g, 0.95 mmol available benzyl alcohol positions) was gently agitated with Fmoc-glycine (311 mg, 1.05 mmol) in dry DMF (8.0 mL) for approximately 15 min prior to the addition of pyridine (461 μL, 5.70 mmol) and 2,6-dichlorobenzoyl chloride (400 μL, 2.85 mmol). The reaction flask was then sealed and gently agitated overnight, after which the resin was filtered under vacuum and washed with DMF (3 × 5 mL) and CH_2Cl_2 (3 × 5 mL). The resin was then dried thoroughly *in vacuo* prior to determination of the loading.

To ascertain the degree of resin loading, 2 small amounts of resin (0.91 and 1.12 mg) were accurately weighed into 2 quartz cuvettes. Freshly made 20% piperidine/DMF (3.0 mL) was then dispensed into the cuvettes along with a third empty cuvette which was used a solvent blank. Each sample was mixed for approximately 5 min and allowed to settle, and the UV absorbance measured to give $A_{(290\ nm)}$ = 0.924 and 1.189, respectively. The loading of the resin was then calculated on the basis of a linear relationship between absorbance at 290 nm and the quantity of Fmoc-amino acid present (with 1 mmol of Fmoc-amino acid giving an absorbance of 1.650), to give loadings of 0.62 and 0.64 meq/g, or an average of 0.63 meq/g.

The remaining benzyl groups of the resin were capped by stirring the resin (1.08 g, 0.32 mmol available benzyl alcohol positions) with pyridine (466 μL, 5.8 mmol) and benzoyl chloride (441 μL, 3.8 mmol) in 1,2-dichloroethane (8 mL) for two hours. The resin was isolated by filtration, washed with 1,2-dichloroethane (3 × 5 mL) and CH_2Cl_2 (3 × 5 mL) and dried *in vacuo*.

2) Peptide synthesis: The synthesis of FmocGFLG-Wang resin was carried out by standard solid phase technique in a glass reaction vessel equipped with a frit (porosity 3) to allow both filtration and agitation of the resin by application of vacuum or positive pressure of nitrogen, respectively. Fmoc-glycine-Wang resin (1.00 g, 0.61 mmol glycine) was allowed to swell in DMF (8 mL) for 15 min, drained, and the Fmoc protecting group removed by reacting the resin with freshly made 20% piperidine/DMF (12 mL) for 10 min. After draining and washing with DMF (3 × 15 mL) and *i*-Pr alcohol (3 × 15 mL) the Kaiser test (for free amines) was carried out as follows:

> *Kaiser Test*: Several beads of the resin were removed and washed with EtOH (3 × 1 mL) in a small glass vial. The following 3 stock solutions were made and 3 drops from each were added to cover the beads:
>
> ninhydrin (2.5 g) in EtOH (50 mL)
> phenol (40 g) in EtOH (10 mL)
> 1 mM aq KCN (2 mL) in pyridine (98 mL).

The vial was capped and placed in an oven (120 °C) for 3 min. A positive result (indicating the presence of free amines) was shown by the beads turning blue, while a negative result (indicating the absence of free amines) was shown by no change in colour (beads remained yellow).

With a positive Kaiser test, indicating the presence of free amines and hence the success of the deprotection reaction, the coupling reaction was carried out. The bulk resin sample was again washed with DMF (3 × 15 mL) and the collection flask changed to remove all traces of piperidine. A solution of Fmoc-L-leucine (431 mg, 1.22 mmol) in DMF (8 mL) was activated for 2 min with HBTU (463 mg, 1.22 mmol) and DIPEA (426 μL, 2.44 mmol), then the resulting solution added to the resin. The coupling reaction was gently agitated for 1 h before draining and washing with DMF (3 × 15 mL). The Kaiser test was again performed to give a negative result, indicating the absence of free amines and thereby the success of the coupling reaction. This process of deprotection and coupling was repeated for Fmoc-L-phenylalanine (473 mg, 1.22 mmol) and Fmoc-glycine (363 mg, 1.22 mmol) with all other reagents used in the same quantities as those described above. Following the final coupling reaction, the resin was washed with DMF (3 × 15 mL), MeOH (3 × 15 mL) and CH_2Cl_2 (3 × 15 mL) before drying *in vacuo*.

3) Cleavage: The *N*-protected tetrapeptide was cleaved from the resin by agitation with a cleavage brew consisting of 95% TFA/2.5% H_2O/2.5% TES (5 mL) for 30 min. The mixture was filtered, then the resin subjected to a second cleavage reaction (5 mL, 10 min) and again filtered. The combined acidic filtrates were pooled, diluted with H_2O (100 mL), to give a white precipitate, and extracted with CH_2Cl_2 (1 × 200 and 2 × 100 mL). The organic fractions were combined, washed with sat brine solution (200 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The yellow oily residues thus obtained were dissolved in THF (20 mL), and the resulting solution diluted with hexanes (200 mL) with vigorous stirring. The precipitate, which formed immediately, was collected by filtration, washed with hexanes and dried *in vacuo* to give the tetrapeptide as an off-white amorphous solid (141 mg, 38% yield).

Mp: 197-199 °C.

^{1}H NMR (500 MHz, d_6-DMSO): δ 0.83 (d, J = 6.3 Hz, 3H, H-16a), 0.87 (d, J = 6.3 Hz, 3H, H-16b) 1.48 (t, J = 7.3 Hz, 2H, H-14), 1.60 (m, 1H, H-15), 2.77 (dd, J= 9.3, 14.2 Hz, 1H, H-6a), 3.02 (dd, J = 4.4, 13.7 Hz, 1H, H-6b), 3.51 (dd, J = 6.3, 17.1 Hz, 1H, H-1a), 3.63 (dd, J = 5.9, 16.6 Hz, 1H, H-1b), 3.70 (dd, J = 6.3, 17.6 Hz, 1H, H-19a), 3.76 (dd, J = 5.9, 17.1 Hz, 1H, H-19b), 4.18-4.27 (m, 3H, H-3' and H-4'), 4.33 (dd, J = 7.8, 15.6 Hz, 1H, H-13), 4.55 (td, J = 4.4,

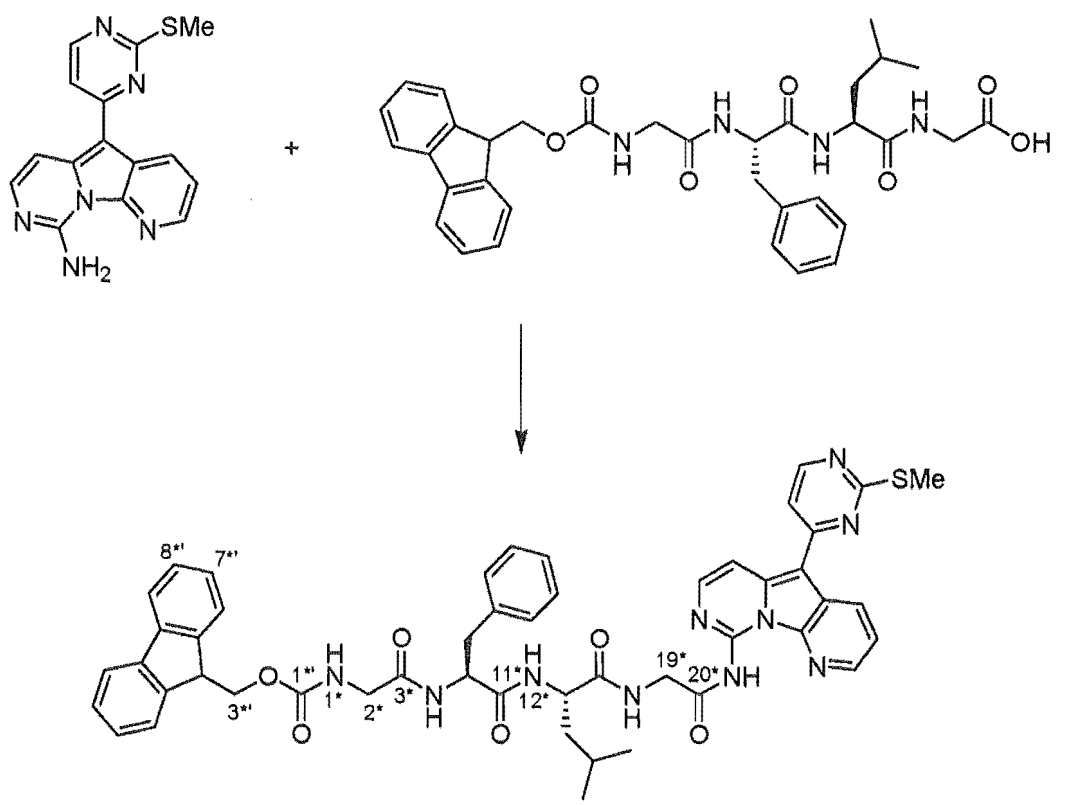
8.6 Hz, 1H, H-5), 7.13-7.18 (m, 1H, H-10), 7.19-7.24 (m, 4H, H-8 and H-9), 7.32 (t, J = 7.3 Hz, 2H, H-7'), 7.41 (t, J = 7.3 Hz, 2H, H-8'), 7.52 (t, J = 5.9 Hz, 1H, H-1), 7.70 (d, J = 7.3 Hz, 2H, H-6'), 7.89 (d, J = 7.3 Hz, 2H, H-9'), 8.00 (d, J = 8.3 Hz, 1H, H-4), 8.09 (t, J = 5.9 Hz, 1H, H-18), 8.12 (d, J = 8.3 Hz, 1H, H-12).

^{13}C NMR (75 MHz, d_6-DMSO): δ 21.6 (C-16a), 23.1 (C-16b), 24.1 (C-15), 37.7 (C-6), 40.4 (C14), 40.9 (C-19), 43.3 (C-2), 46.6 (C-4'), 51.1 (C-13), 53.9 (C-5), 65.8 (C-3'), 120.1 (C-9'), 125.3 (C-6'), 126.3 (C-10), 127.1 (C-7'), 127.7 (C-8'), 128.0 (C-9), 129.3 (C-8), 137.8 (C-7), 140.7 (C-10'), 143.9 (C-5'), 156.5 (C-1'), 169.0 (C-3), 170.9 (C-11), 171.2 (C-20), 171.6 (C-17).

IR (KBr): υ_{max} 3275, 3055, 2938, 1712, 1647, 1545, 1452, 1404, 1348, 1250, 1169, 1088, 1061, 935, 741, 702.

HRMS (ESI): Calcd for $C_{34}H_{39}N_4O_7$ (MH^+) *m/z* = 615.2819, found *m/z* = 615.2816.

9-[*N*-(9-Fluorenylmethoxycarbonyl)glycylphenylalanylleucylglycylamido]-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.18



A solution of variolin **2.16** (10.0 mg, 32 μmol), FmocGFLG (19.9 mg, 32 μmol), DCC (11.7 mg, 57 μmol) and HOBT (8.8 mg, 65 μmol) in THF (2.0 mL) was left at 4 °C for 4 d, during which time a yellow gel-like precipitate formed. The precipitate was collected by filtration, washed with CH_2Cl_2 (1 mL), MeOH (1 mL) and dried *in vacuo* to afford the conjugate, with 5-10 mol% contamination by DCU, as a yellow amorphous solid (8.6 mg, 95% pure, 28% yield). (All attempts at further purification of the conjugate material, including the significant amount that remained in solution when the reaction was complete, were hindered by decomposition of the conjugate.)

^{1}H NMR (500 MHz, d_6-DMSO): δ 0.86 (d, J = 6.8 Hz, 3H, H-16*a), 0.87 (d, J = 6.3 Hz, 3H, H-16*b), 1.52-1.67 (m, 3H, H-14* and H-15*), 2.59 (s, 3H, SC*H*$_3$), 2.78 (dd, J= 8.9, 14.2 Hz, 1H, H-6*a), 3.02 (dd, J = 4.7, 14.2 Hz, 1H, H-6*b), 3.52 (dd, J = 5.8, 16.8 Hz, 1H, H-2*a), 3.66 (dd, J = 6.3, 16.8 Hz, 1H, H-2*b), 4.15-4.25 (m, 3H, H-3*' and H-4*'), 4.40-4.53 (m, 3H, H-13*, H-19*a and H-19*b), 4.57 (td, J = 4.2, 8.4 Hz, 1H, H-5*), 7.13-7.18 (m, 1H, H-10*), 7.19-7.24 (m, 4H, H-8* and H-9*), 7.28 (t, J = 7.4 Hz, 2H, H-7*'), 7.36 (t, J = 7.4 Hz, 2H, H-8*'), 7.49-7.55 (m, 2H, H-5' and H-1*), 7.62 (dd, J = 4.2, 7.9 Hz, 1H, H-3), 7.67 (d, J = 7.4 Hz, 2H, H-6*'), 7.75 (d, J = 6.8 Hz, 1H, H-7), 7.82 (d, J = 7.9 Hz, 2H, H-9*'), 7.91 (d, J = 6.8 Hz, 1H, H-6), 8.06 (d, J = 7.9 Hz, 1H, H-4*), 8.20 (d, J = 8.4 Hz, 1H, H-12*), 8.37 (t, J = 5.3 Hz, 1H, H-18*), 8.51-8.54 (m, 2H, H-2 and H-6'), 8.75 (d, J = 7.9, 1H, H-4), 12.87 (s, 1H, 9-N*H*).

^{13}C NMR (75 MHz, d_6-DMSO): δ 13.9 (SCH$_3$), 21.8 (C-16*a), 23.2 (C-16*b), 24.2 (C-15*), 35.7 (C-6*), 41.0 (C-14*), 43.4 (C-2*), 45.4 (C-19*), 46.7 (C-4*'), 51.2 (C-13*), 54.0 (C-5*), 65.9 (C-3*'), 100.4 (C-5), 107.8 (C-6), 113.3 (C-5'), 120.2 (C-9*'), 120.9 (C-4a), 121.6 (C-3), 125.4 (C-6*'), 126.4 (C-10*'), 127.2 (C-7*'), 127.8 (C-8*'), 128.2 (C-9*), 129.4 (C-10*), 129.9 (C-4), 137.75 (C-5a or C-7*), 137.77 (C-5a or C-7*), 140.8 (C-10*'), 141.39 (C-2 or C-7), 141.47 (C-2 or C-7), 142.1 (C-10a), 143.0 (C-9), 143.9 (C-5*'), 156.7 (C-1*'), 157.5 (C-6'), 160.3 (C-4'), 169.2 (C-3*), 169.6 (C-20*), 171.0 (C-11*), 171.4 (C-2'), 172.8 (C-17*).

IR (KBr): υ_{max} 3348, 2965, 1735, 1669, 1587, 1554, 1510, 1475, 1366, 1243, 1205, 1046.

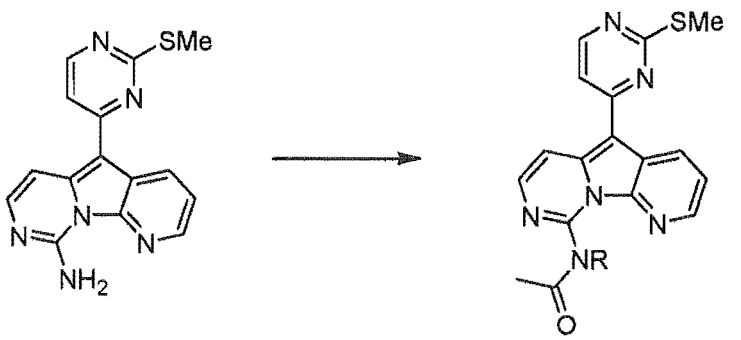
HRMS (ESI): Calcd for $C_{49}H_{49}N_{10}O_6{}^{32}S$ (MH^+) *m/z* = 905.3557, found *m/z* = 905.3556.

IC_{50} (P388): >13.8 μM

9-(*N*-Acetamido)-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.19

and

9-(*N*,*N*-Diacetimido)-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.20



2.19: R = H
2.20: R = $COCH_3$

a) To a stirred solution of variolin amine **2.16** (14.0 mg, 45 μmol) and TEA (35 μL, 250 μmol) in THF (5.0 mL) was slowly added AcCl (16 μL, 227 μmol), and the solution stirred at rt. The solution darkened significantly from yellow to orange during the first 15 min. After 44 h the reaction solution was concentrated *in vacuo*, and the yellow/brown residues thus obtained purified by repeated flash chromatography on neutralised silica, eluting with 1-2.5% IPA/CH_2Cl_2 (0.05% TEA), to afford the monoacetylated derivative **2.19** (5.7 mg, 36% yield) and diacetylated derivative **2.20** (4.5 mg, 25% yield) as yellow microcrystalline solids.

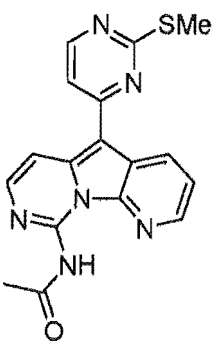
b) To a stirred solution of variolin amine **2.16** (20.0 mg, 65 μmol) and TEA (50 μL, 359 μmol) in THF (3.0 mL) was slowly added AcCl (23 μL, 323 μmol), and the solution stirred at rt. The solution darkened significantly from yellow to orange during the first 15 min. Aliquots (30 μL) were periodically taken, dried *in vacuo*, and examined by ^{1}H NMR spectroscopy.

c) To a stirred solution of variolin amine **2.16** (20.0 mg, 65 μmol) and TEA (50 μL, 359 μmol) in THF (3.0 mL) was slowly added AcCl (23 μL, 323 μmol), and the solution stirred at rt for 19 h, during which time the solution had darkened significantly from yellow to orange. At this time an aliquot (25 μL) was removed, dried *in vacuo*, and examined by ^{1}H NMR spectroscopy.

d) To a stirred solution of variolin amine **2.16** (20.0 mg, 65 μmol) and TEA (14 μL, 97 μmol) in THF (3.0 mL) was slowly added AcCl (4.6 μL, 65 μmol), and the solution stirred at rt for 19 h, during which time the solution darkened significantly from yellow to orange. An aliquot (25 μL) was removed, dried *in vacuo*, and examined by ^{1}H NMR spectroscopy.

e) To a stirred solution of variolin amine **2.16** (22.0 mg, 71 μmol) and TEA (25 μL, 178 μmol) in THF (5.0 mL) was slowly added AcCl (10 μL, 143 μmol), and the solution stirred at rt. The solution darkened significantly from yellow to orange during the first 15 min. Aliquots (10 μL) were periodically taken, diluted with THF (100 μL), and examined by HPLC at 256 nm, eluting with 85% MeOH/H_2O (0.05% TFA).

f) To a stirred solution of variolin amine **2.16** (20.0 mg, 65 μmol) and TEA (14 μL, 97 μmol) in THF (6.0 mL) was slowly added AcCl (4.6 μL, 65 μmol), and the solution stirred at rt for 48 h, during which time the solution darkened significantly from yellow to orange. The solution was then concentrated *in vacuo* and the yellow/brown residues dissolved in CH_2Cl_2 (15 mL), washed with sat aq $NaHCO_3$ (15 mL), sat brine solution (15 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by flash chromatography on silica, eluting with 0.5-5% MeOH/CH_2Cl_2, to give the monoacetylated derivative **2.19** (0.9 mg, 4% yield), diacetylated derivative **2.20** (7.2 mg, 28% yield) and variolin starting material **2.16** (6.5 mg, 33% recovery) as yellow microcrystalline solids.



Mp: 203-205 °C.

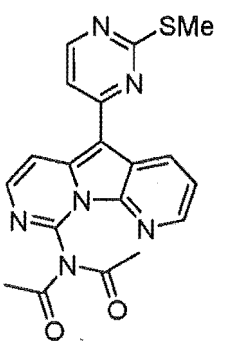
^{1}H NMR (500 MHz, $CDCl_3$): δ 2.676 (s, 3H, $COCH_3$), 2.684 (s, 3H, SCH_3), 7.35 (d, J = 5.4 Hz, 1H, H-5'), 7.58 (dd, J = 4.4, 8.3 Hz, 1H, H-3), 7.83 (d, J = 6.8 Hz, 1H, H-7), 7.93 (d, J = 6.8 Hz, 1H, H-6), 8.52 (dd, J = 1.5, 4.4 Hz, 1H, H-2), 8.54 (d, J = 5.4 Hz, 1H, H-6'), 8.77 (dd, J = 1.5, 8.3 Hz, 1H, H-4).

^{13}C NMR (75 MHz, $CDCl_3$): δ 14.3 (SCH_3), 26.5 ($COCH_3$), 101.4 (C-5), 107.4 (C-6), 112.9 (C-5'), 121.1 (C-3), 121.6 (C-4a), 129.5 (C-4), 137.7 (C-5a), 141.0 (C-2), 141.2 (C-7), 142.5 (C-10a), 143.3 (C-9), 156.9 (C-6'), 160.7 (C-4'), 170.2 (*C*O), 172.7 (C-2').

IR (KBr): υ_{max} 1867, 1682, 1568, 1456, 1273, 1202, 1132, 1030, 800, 768, 721, 552.

HRMS (ESI): Calcd for $C_{17}H_{15}N_6O^{32}S$ (MH^+) *m/z* = 351.1028, found *m/z* = 351.1027.

IC_{50}: (P388) 12.6 μM.



Mp: 166-169 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.41 (s, 6H, $CO(CH_3)_2$), 2.68 (s, 3H, SCH_3), 7.34 (d, J = 5.4 Hz, 1H, H-5'), 7.53 (dd, J = 4.9, 8.3 Hz, 1H, H-3), 7.90 (d, J = 6.8 Hz, !H, H-7), 8.36 (d, J = 6.3 Hz, 1H, H-6), 8.53 (dd, J = 1.5, 4.4 Hz, 1H, H-2), 8.56 (d, J = 5.4 Hz, 1H, H-6'), 8.62 (dd, J = 1.5, 8.3 Hz, 1H, H-4).

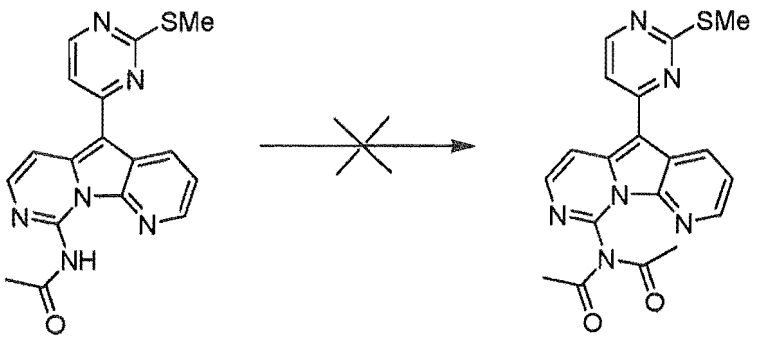
^{13}C NMR (75 MHz, $CDCl_3$): δ 14.3 (SCH_3), 25.8 ($COCH_3$), 103.4 (C-5), 113.4 (C-5'), 114.2 (C-6), 121.0 (C-4a), 121.8 (C-3), 128.9 (C-4), 137.2 (C-5a), 139.2 (C-7), 141.5 (C-9), 141.8 (C-10a), 144.2 (C-2), 157.1 (C-6'), 160.3 (C-4'), 171.4 (C-2'), 172.9 (*C*O).

IR (KBr): υ_{max} 1684, 1567, 1456, 1273, 1203, 1132, 1030, 800.

HRMS (ESI): Calcd for $C_{19}H_{17}N_6O_2{}^{32}S$ (MH^+) *m/z* = 393.1134, found *m/z* = 393.1147.

IC_{50}: (P388) 16.1 μM.

Attempted acetylation of monoacetylated variolin 2.19



a) To a stirred solution of monoacetylated variolin **2.19** (15.0 mg, 43 μmol) and TEA (33 μL, 237 μmol) in 1:1 THF/CH_2Cl_2 (5.0 mL) was slowly added AcCl (15 μL, 211 μmol) and the solution stirred at rt for 22 h. During this time the solution darkened significantly from yellow to orange. The solution was then concentrated *in vacuo*, examined by ^{1}H NMR spectroscopy, and purified by flash chromatography on neutralised silica, eluting with 1% MeOH/CH_2Cl_2 (0.1% TEA), to give the monoacetylated starting material **2.19** as a yellow microcrystalline solid (13.7 mg, 81% recovery).

b) To a stirred solution of monoacetylated variolin **2.19** (15 mg, 43 μmol), bis-SMe analogue **2.6** (14.0 mg, 41 μmol) and TEA (33 μL, 237 μmol) in THF (10 mL) was slowly added AcCl (15 μL, 211 μmol) and the solution stirred at rt for 48 h. During this time the solution darkened significantly from yellow to orange. An aliquot (50 μL) was removed, concentrated *in vacuo* and examined by ^{1}H NMR spectroscopy.

Buffers

Acetate buffer, pH = 5.0 (0.02 M):

Acetic acid (50 μL) and $NaCOCH_3$ (82 mg) were dissolved in H_2O (100 mL), and $NaCOCH_3$ (~100 mg) added to adjust the pH to 5.0.

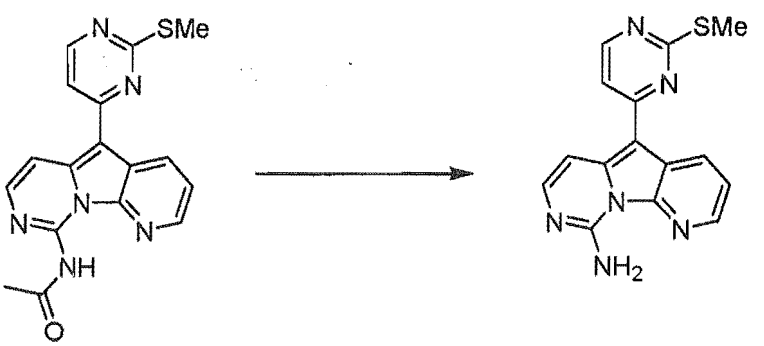
Phosphate buffer, pH = 7.4 (0.04 M):

KH_2PO_4 (118 mg) and $Na_2HPO_4.12H_2O$ (1.09 g) were dissolved in H_2O (100 mL), and a small amount of KH_2PO_4 was added adjust the pH to 7.4.

Borax buffer, pH = 9.3 (0.01 M):

$Na_2B_4O_7.12H_2O$ (381 mg) was dissolved in H_2O (100 mL).

Decomposition of monoacetylated variolin 2.19



a) To a solution of monoacetylated variolin **2.19** (5.0 mg, 14 μmol) in 1:1 $MeOH/CH_2Cl_2$ (2.0 mL) was added piperidine (2 μL, 20 μmol), and the reaction analysed by tlc, eluting with 5% $MeOH/CH_2Cl_2$, after 30 min and 2h. Piperidine (20 μL, 201 μmol) was again added, and the reaction analysed by tlc as before. Finally, piperidine (200 μL, 2.0 mmol) was again added, and the reaction analysed by tlc as before.

b) To a solution of monoacetylated variolin **2.19** (5.0 mg, 14 μmol) in 1:1 $MeOH/CH_2Cl_2$ (2.0 mL) was added TFA (2 μL, 26 μmol), and the reaction analysed by tlc, eluting with 5% $MeOH/CH_2Cl_2$, after 30 min and 2h. TFA (20 μL, 260 μmol) was again added, and the reaction analysed by tlc after 30 min.

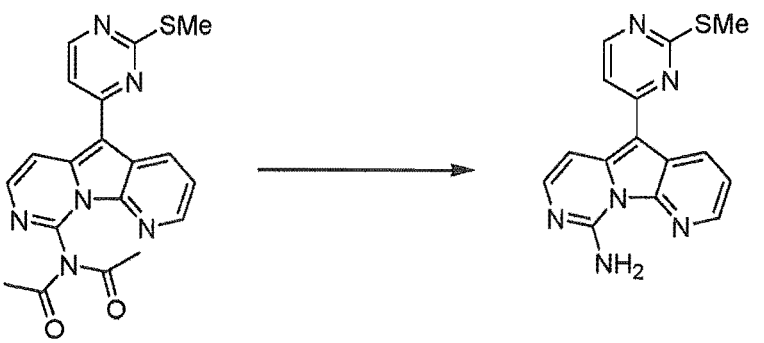
c) Monoacetylated variolin **2.19** (7.0 mg, 20 μmol) was dissolved in triflic acid (500 μL) and the deep orange/yellow solution stirred at rt for 22 h. The solution was cooled to 0 °C and, with stirring, neutralised with aq NH_3 (4 mL). Sat aq $NaHCO_3$ solution (10 mL) was added, and the

resulting yellow suspension extracted with CH_2Cl_2 (10 mL). The yellow organic solution was washed with sat brine solution (10 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo* to afford free amine **2.16** as a yellow/brown amorphous solid (4.4 mg, 95% pure, 76% yield).

d) The following manipulations were carried out separately with pH = 5.0 acetate, pH = 7.4 phosphate and pH = 9.3 borax buffers. A solution of monoacetylated variolin **2.19** in DMSO (29 μM, 1.2 mL) was diluted with aqueous buffer solution (0.3 mL) and left at 30 °C. Aliquots (100 μL) were periodically removed and analysed by HPLC (5 μL injections) at 256 nm, eluting with 85% MeOH/H_2O.

e) The protocols described in part d) were replicated, with the exceptions that a 2% DMSO/THF organic solution was used (in the place of DMSO) and reactions were only analysed once after 43 h.

Decomposition of diacetylated variolin 2.20



a) To a solution of diacetylated variolin **2.20** (5.0 mg, 13 μmol) in 1:1 MeOH/CH_2Cl_2 (2.0 mL) was added piperidine (2 μL, 20 μmol), and the reaction analysed by tlc, eluting with 5% MeOH/CH_2Cl_2, after 30 min and 2h. Piperidine (20 μL, 201 μmol) was again added, and the reaction analysed by tlc after 30 min.

b) To a solution of diacetylated variolin **2.20** (5.0 mg, 14 μmol) in 1:1 MeOH/CH_2Cl_2 (2.0 mL) was added TFA (2 μL, 26 μmol), and the reaction analysed by tlc, eluting with 5% MeOH/CH_2Cl_2, after 30 min and 2h. TFA (20 μL, 260 μmol) was again added, and the reaction

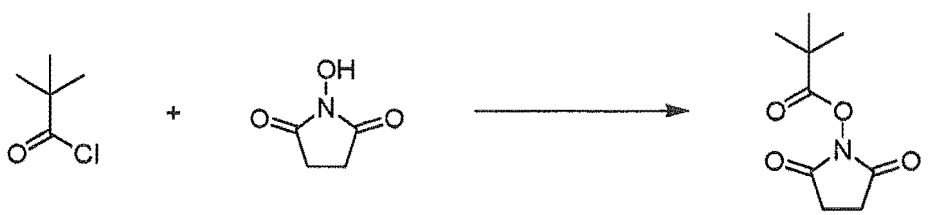
analysed by tlc as before. Finally, TFA (200 μL, 2.6 mmol) was again added, and the reaction analysed by tlc after 30 min.

Pivaloyl chloride

This was prepared from pivalic acid,[139] following the procedure of Brown.[140] A solution of pivalic acid (74.2 mL, 431 mmol) in benzoyl chloride (100 mL, 861 mmol) was fractionally distilled, with the fraction boiling at 60-80 °C being collected. This material was again fractionally distilled, and the fraction boiling at 92 °C collected to give the title compound (50.3 g, 64% yield).

Bp: 92 °C (lit. 93 °C).[139]

***N*-Hydroxysuccinimidyl pivalate 2.23**

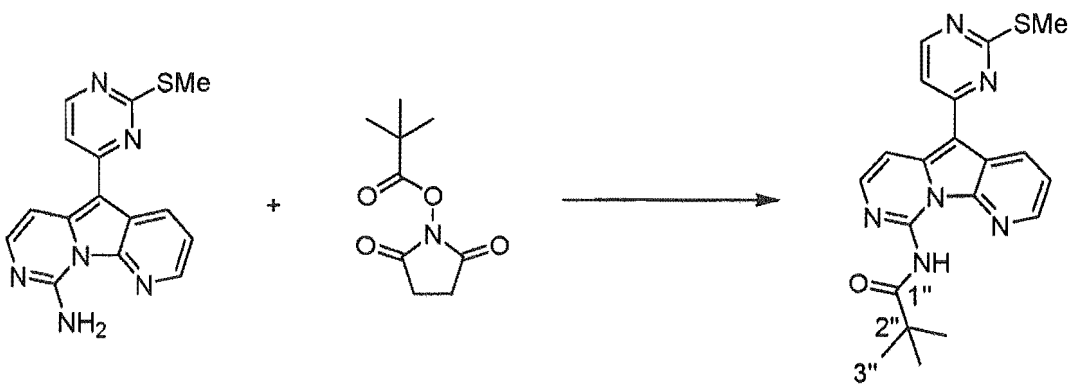


To a stirred solution of NHS (1.00 g, 8.7 mmol) and TEA (1.39 mL, 10.0 mmol) in CH_2Cl_2 (50 mL) at rt was added pivaloyl chloride (1.18 mL, 9.6 mmol) dropwise over 10 min, and the solution left stirring at rt for 1 h. The resulting white suspension was washed with 0.2 M aq HCl (50 mL), sat aq $NaHCO_3$ (50 mL), sat brine solution (50 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was recrystallised from benzene/hexanes to afford the title compound as white needles (1.45 g, 83% yield).

Mp: 77-78 °C (benzene) (lit. 77-8 °C).[108]

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.39 (s, 9H, CH_3), 2.83 (d, J = 5.4 Hz, 4H, $CH2$).

9-(*N*-pivalamido)-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.24



a) A solution of variolin analogue **2.16** (10.0 mg, 32 μmol) and ester **2.23** (13.0 mg, 64 μmol) in DMSO (0.7 mL) was heated at 100 °C for 19 h. The yellow solution was allowed to cool to rt, diluted with CH_2Cl_2 (10 mL), washed with sat brine solution (15 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by repeated flash chromatography on neutralised silica, eluting with 1% MeOH/CH_2Cl_2 (0.1% TEA), to afford both the variolin starting material **2.16** as a yellow microcrystalline solid (5.1 mg, 51% yield) and the acylated conjugate **2.24** in low purity. This conjugate material was then purified by flash chromatography on silica, eluting with 10-50% EtOAc/CH_2Cl_2, to afford the acylated variolin product as a yellow powder (3.0 mg, 24% yield).

b) A solution of variolin **2.16** (7.6 mg, 25 μmol) and ester **2.23** (49.1 mg, 246 μmol) in DMSO (0.7 mL) was heated at 110 °C for 72 h. The yellow solution was allowed to cool to rt, diluted with CH_2Cl_2 (10 mL), washed with sat brine solution (15 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by flash chromatography on neutralised silica, eluting with 1% MeOH/CH_2Cl_2 (0.1% TEA), to afford the acylated variolin product as a yellow powder (3.0 mg, 31% yield).

Mp: >230 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.50 (s, 9H, H-3"), 2.69 (s, 3H, SCH_3), 7.36 (d, J = 5.4 Hz, 1H, H-5'), 7.59 (dd, J = 4.4, 8.3 Hz, 1H, H-3), 7.93 (d, J = 6.8 Hz, 1H, H-7), 7.95 (d, J = 6.8 Hz, 1H,

H-6), 8.50 (dd, J = 1.5, 4.4 Hz, 1H, H-2), 8.54 (d, J = 5.4 Hz, 1H, H-6'), 8.81 (dd, J = 1.5, 8.3 Hz, 1H, H-4), 13.37 (s, 1H, 9-N*H*).

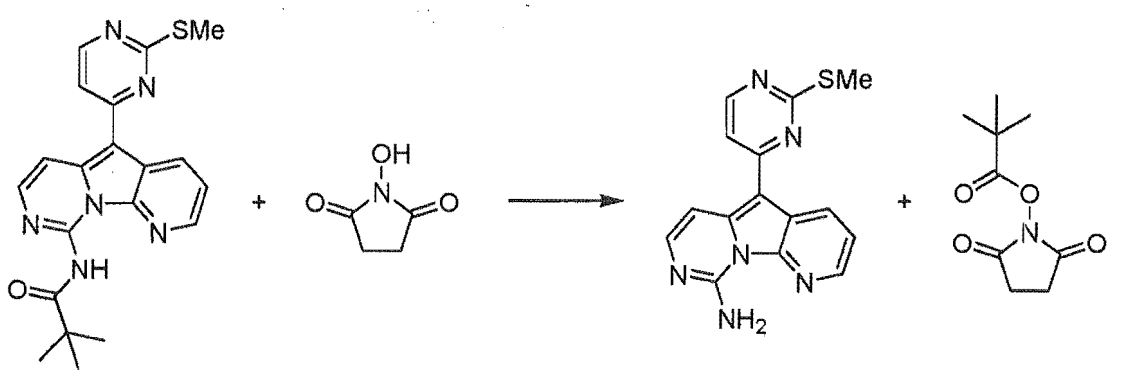
^{13}C NMR (75 MHz, $CDCl_3$): δ 14.3 (SCH_3), 27.3 (C-3''), 41.4 (C-2''), 101.3 (C-5), 107.5 (C-6), 112.9 (C-5'), 121.1 (C-3), 121.6 (C-4a), 129.8 (C-4), 138.0 (C-5a), 140.4 (C-2), 142.0 (C-7), 142.5 (C-10a), 143.7 (C-9), 156.8 (C-6'), 160.8 (C-4'), 172.7 (C-2'), 176.4 (C-1'').

IR (KBr): υ_{max} 1722, 1630, 1558, 1520, 1497, 1477, 1396, 1362, 1335, 1246, 1165, 1070, 1007, 910, 831, 806, 770, 741, 687.

HRMS (ESI): Calcd for $C_{20}H_{21}N_6O^{32}S$ (MH^+) *m/z* = 393.1498, found *m/z* = 393.1494.

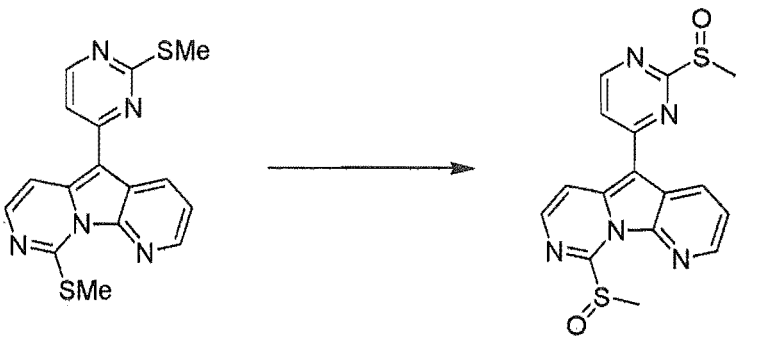
IC_{50} (P388): >8.0 μM.

Ester 2.23 from acylated variolin 2.24



A solution of variolin **2.24** (2.0 mg, 5 μmol) and NHS (0.6 mg, 5 μmol) in DMSO (0.5 mL) was heated to 100 °C for 48 h. The yellow solution was allowed to cool to rt, diluted with CH_2Cl_2 (10 mL), washed with H_2O (2 × 15 mL), sat brine solution (15 mL), dried with Na_2SO_4, filtered, concentrated *in vacuo* and examined by ^{1}H NMR spectroscopy. The crude material thus obtained was purified by flash chromatography on neutralised silica, eluting with 1% MeOH/CH_2Cl_2 (0.1% TEA), to afford the NHS ester product **2.23** (50% pure, yield not taken) and both the variolin starting material **2.24** and product **2.16** in high purity (yields not taken).

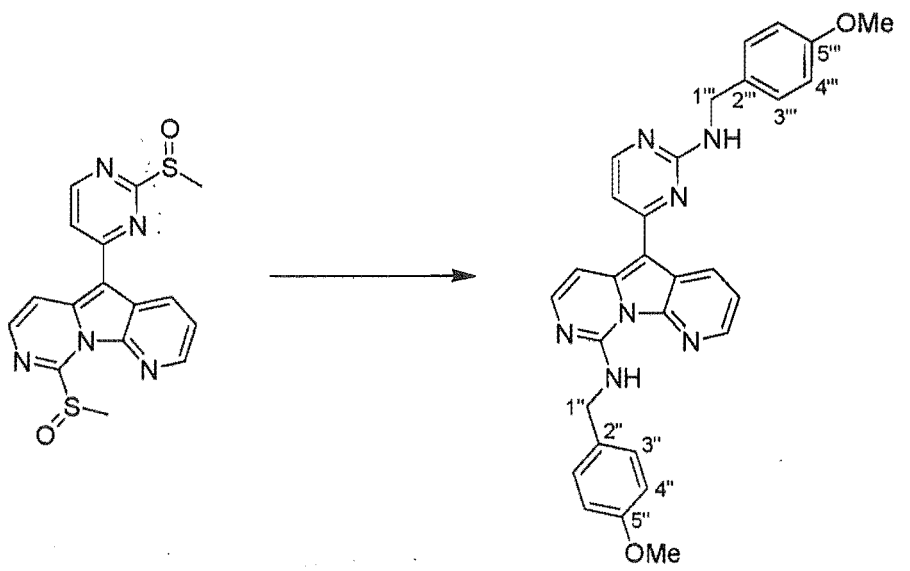
9-(Methylsulfinyl)-5-[2-(methylsulfinyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.25[87]



Under atmospheric conditions, a solution of *m*-CPBA (102 mg, 591 μmol) in chloroform (10 mL) was cooled to -55 °C (MeOH/N_2), and added dropwise, over 15 min, to a similarly cooled stirred solution of core structure **2.6** (100 mg, 295 μmol) in chloroform (10 mL). The solution was left to warm to ~0 °C over 1 h then washed with sat aq $NaHCO_3$ solution (25 mL), sat brine solution (25 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo* to leave a yellow solid. This crude material, which was used without further purification, was predominantly a mixture of diastereomeric bis-sulfoxides with the following spectroscopic characteristics.

^{1}H NMR (500 MHz, $CDCl_3$): (As most signals for the diastereoisomers coincide, some are quoted as multiplets.) δ 3.04 (d, 3H, 2'-SC*H*$_3$), 3.24 (d, 3H, 9-SC*H*$_3$), 7.65 (dd, 1H), 7.79 (d, 1H), 8.24 (m, 1H), 8.64 (m, 1H), 8.69 (m, 1H), 8.80 (m, 1H), 8.86 (d, 1H).

9-(4-Methoxybenzylamino)-5-[2-(4-methoxybenzylamino)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.26[87]



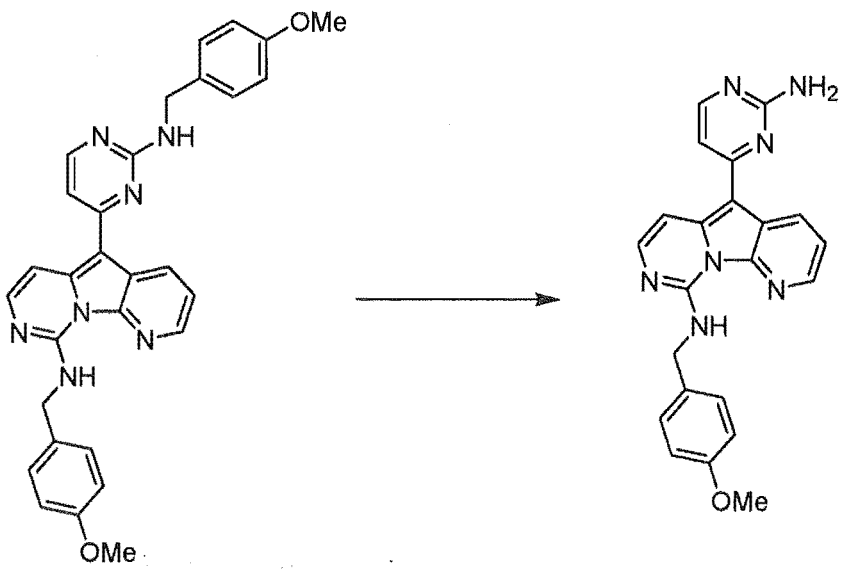
Oxidised core structure **2.25** (109 mg, 0.29 mmol, if oxidation reaction gave a quantitative yield) was heated with *p*-methoxybenzylamine (192 μL, 1.46 mmol) in toluene (2 mL) at 100 °C for 3 d. The solution was allowed to cool to rt then diluted with CH_2Cl_2 (20 mL), washed with sat aq $NaHCO_3$ solution (20 mL), sat brine solution (20 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by repeated flash chromatography on neutralised silica, eluting with 0.5% MeOH/DCM (0.1% TEA), to give the title compound as an orange foam (109 mg, 72% yield over 2 steps).

Mp: 58-62 °C (lit. 54-60 °C).[87]

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.801 (s, 3H, OC*H*$_3$), 3.805 (s, 3H, OC*H*$_3$), 4.68 (d, J = 5.9 Hz, 2H, H-1'''), 4.88 (d, J = 5.4 Hz, 2H, H-1''), 5.58 (br, 1H, 2'-N*H*), 6.9 (m, 4H, H-4'' and H-4'''), 6.97 (d, J = 5.4 Hz, 1H, H-5'), 7.33-7.42 (m, 6H, H-3, H-6, H-3'' and H-3'''), 7.62 (d, J = 6.3 Hz, 1H, H-7), 8.26 (dd, J = 1.5, 4.9 Hz, 1H, H-2), 8.29 (d, J = 5.4 Hz, 1H, H-6'), 8.55 (br d, J = 7.8 Hz, 1H, H-4), 10.35 (t, J = 5.9 Hz, 1H, 9-N*H*).

IC_{50} (P388): >34 μM

9-(4-Methoxybenzylamino)-5-[2-(amino)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.27[87]



Bis-PMB-protected variolin **2.26** (20.0 mg, 39 μmol) was dissolved in TFA (0.5 mL) and stirred at 45 °C for 24 h. The deep red solution was cooled to 0 °C then neutralised by the dropwise addition of aq NH_3. Sat aq $NaHCO_3$ solution was added until effervescing ceased. The yellow suspension was then extracted with CH_2Cl_2 (10 mL). The yellow organic solution was washed with sat brine solution (15 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by flash chromatography on neutralised silica, eluting with 2% MeOH/CH_2Cl_2 (0.1% TEA), to give the monoprotected product as a yellow microcrystalline solid (12.8 mg, 83% yield).

Mp: 192-194 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.80 (s, 3H, OCH_3), 4.88 (d, J = 5.4 Hz, 2H, H-1"), 5.14 (br s, 2H, 2'-NH_2), 6.91 (dt, J = 2.0, 8.8 Hz, 2H, H-4"), 6.99 (d, J = 5.4 Hz, 2H, H-5'), 7.38 (dd, J = 4.4, 7.8 Hz, 1H, H-3), 7.41 (dt, J = 8.8, 2.9 Hz, 2H, H-3"), 7.45 (d, J = 6.8 Hz, 1H, H-6), 7.68 (d, J = 6.8 Hz, 1H, H-7), 8.26 (dd, J = 1.5, 4.4 Hz, 1H, H-2), 8.28 (d, J = 5.4 Hz, 1H, H-6'), 8.64 (dd, J = 1.5, 8.3 Hz, 1H, H-4), 10.34 (t, J = 5.4 Hz, 1H, 9-N*H*).

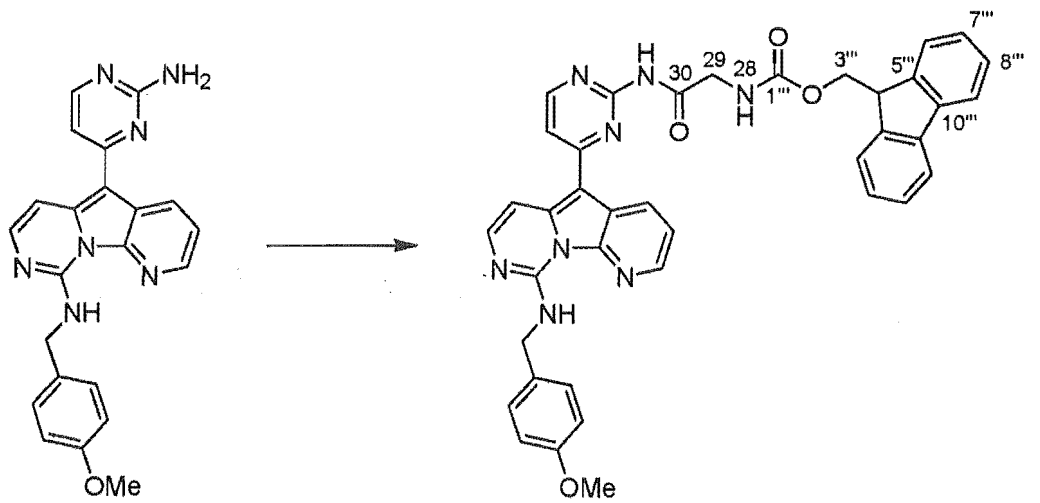
^{13}C NMR (75 MHz, $CDCl_3$): δ 44.4 (C-1"), 55.3 (OCH_3), 100.2 (C-5), 101.4 (C-6), 108.8 (C-5'), 114.1 (C-4"), 120.1 (C-3), 121.9 (C-4a), 128.3 (C-4), 128.8 (C-3"), 130.3 (C-2"), 138.5 (C-5a), 139.6 (C-2), 143.22 (C-7), 143.39 (C-10a), 149.1 (C-9), 157.6 (C-6'), 159.0 (C-5"), 162.5 (C-4'), 162.9 (C-2').

IR (KBr): υ_{max} 1565, 1508, 1459, 1420, 1250, 1175, 1038, 1008, 806, 768, 705.

HRMS (ESI): Calcd for $C_{22}H_{20}N_7O$ (MH^+) *m/z* = 398.1729, found *m/z* = 398.1726.

IC_{50} (P388): 5.4 μM

9-(4-Methoxybenzylamino)-5-[2-{*N*-(9-fluorenylmethoxycarbonyl)glycyl}pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.28



a) PMB-protected variolin **2.27** (19.9 mg, 50 μmol), Fmoc-glycine acid chloride (15.8 mg, 50 μmol) and DMAP (0.2 mg, 1.6 μmol) were disolved in THF (4.0 mL) and TEA (35 μL, 252 μmol) immediately added to give an orange solution with a fine colourless precipitate. After stirring at rt for 20 h the reaction was concentrated *in vacuo*. The crude material thus obtained was purified by flash chromatography on neutralised silica, eluting with 1% MeOH/DCM (0.1% TEA), to give the monoacylated variolin analogue as a thick yellow gum (4.8 mg, 14% yield).

b) PMB-protected variolin **2.27** (29.8 mg, 75 μmol), Fmoc-glycine acid chloride (95.4 mg, 302 μmol) and DMAP (0.4 mg, 3 μmol) were disolved in THF (5.0 mL) and TEA (52 μL, 377 μmol) immediately added to give an orange solution with a fine colourless precipitate. After stirring at rt for 16 h the reaction was examined by tlc, and shown to contain a mixture of variolin compounds in similar proportions to the previous experiment. Concentration of the reaction mixture *in vacuo* gave an orange oil, which was purified by flash chromatography on neutralised silica, eluting with 1% MeOH/DCM (0.1% TEA), to give the monoacylated variolin analogue as a thick yellow gum (yield not taken).

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.81 (s, 3H, OC*H*$_3$), 4.25 (t, J = 6.7 Hz, 1H, H-4'''), 4.43 (d, J = 6.7 Hz, 2H, H-3'''), 4.57 (br s, 2H, H-29), 4.89 (d, J = 4.8 Hz, 2H, H-1''), 5.86 (br, 1H, H-14), 6.92 (d t, J = 2.6, 6.3 Hz, 2H, H-4''), 7.28-7.32 (m, 3H, H-5' and H-7'''), 7.36-7.43 (m, 5H, H-3, H-3'' and H-8'''), 7.54 (d, J = 6.3 Hz, 1H, H-6), 7.62 (d, J = 7.1 Hz, 2H, H-6'''), 7.74 (d, J = 7.5 Hz, 2H, H-9'''), 7.77 (d, J = 6.7 Hz, 1H, H-7), 8.27 (d, J = 4.4 Hz, 1H, H-2), 8.48 (d, J = 5.2 Hz, 1H, H-6'), 8.74 (d, J = 7.9 Hz, 1H, H-4), 8.81 (br s, 1H, 2'-N*H*), 10.40 (br, 1H, 9-N*H*).

^{13}C NMR (75 MHz, $CDCl_3$): δ 44. (C-1''), 45.8 (C-29), 47.1 (C-4'''), 55.3 (O*C*H$_3$), 67.3 (C-3'''), 99.5 (C-5), 101.3 (C-6), 112.7 (C-5'), 114.1 (C-4''), 119.9 (C-9'''), 120.6 (C-3), 121.8 (C-4a), 125.1 (C-6'''), 127.0 (C-7'''), 127.6 (C-8'''), 128.4 (C-4), 128.9 (C-3''), 130.1 (C-2''), 139.6 (C-5a), 139.9 (C-2), 141.2 (C-10'''), 143.60 (C-10a), 143.84 (C-5'''), 144.6 (C-7), 149.2 (C-9), 156.77 (C-1''' and C-2'), 157.2 (C-6'), 159.0 (C-5''), 162.7 (C-4'), 170.1 (C-30).

HRMS (ESI): Calcd for $C_{39}H_{33}N_8O_4$ (MH^+) *m/z* = 677.2625, found *m/z* = 677.2626.

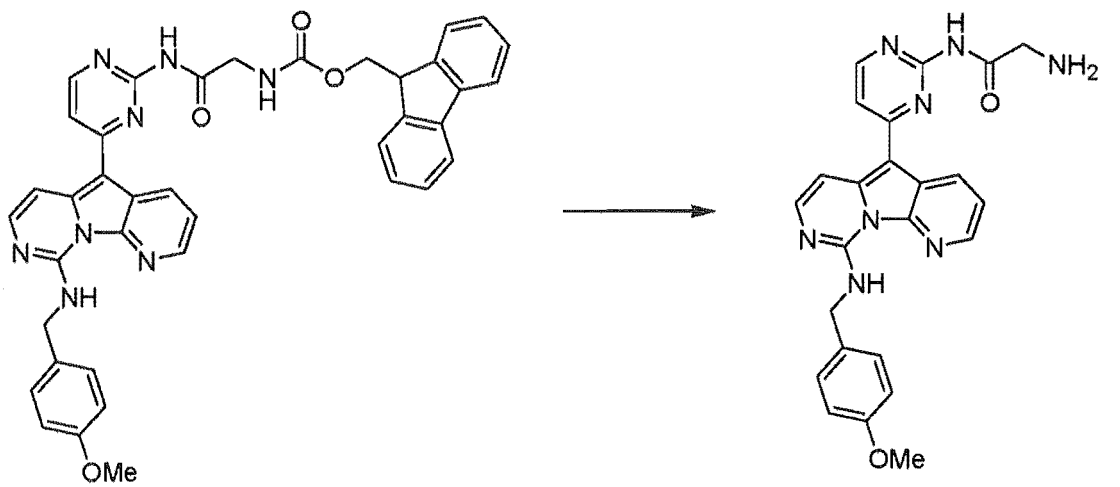
Testing stability of variolin analogue 2.28 towards TFA

An orange solution of variolin analogue **2.28** (~1 mg) in 10% TFA/DMSO (0.5 mL) was stirred at rt for 5 h, then examined by HPLC.

Testing stability of variolin analogue 2.28 towards TEA

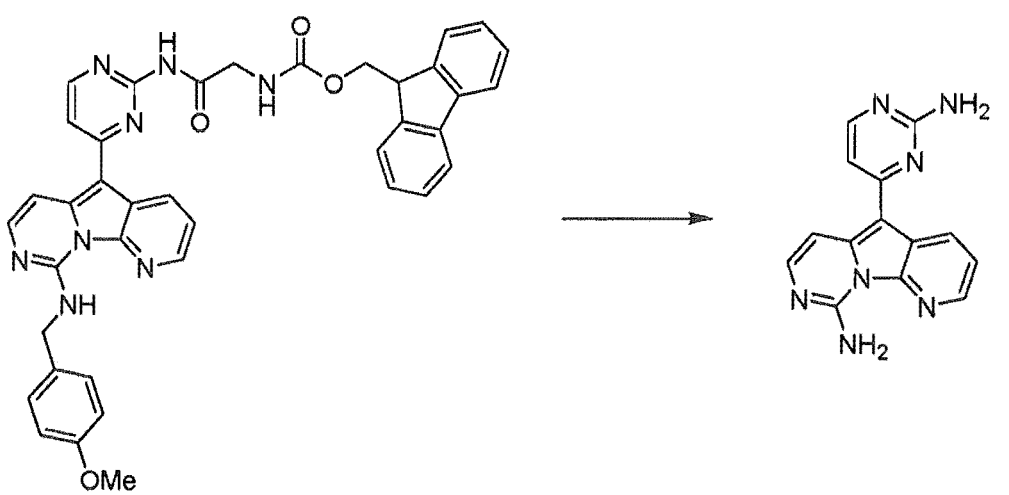
An orange solution of variolin analogue **2.28** (~1 mg) in 10% TEA/DMSO (0.5 mL) was stirred at rt for 5 h, then examined by HPLC.

9-(4-Methoxybenzylamino)-5-[2-(glycyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.29



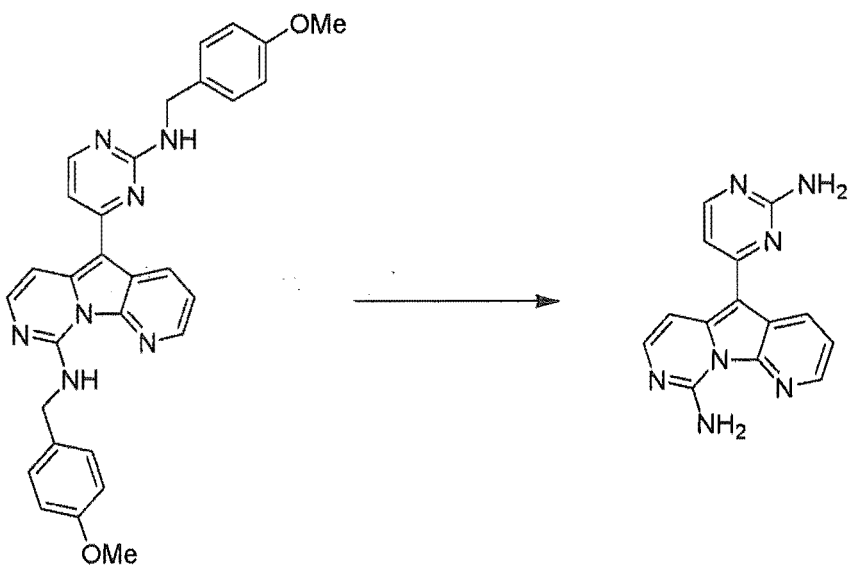
Variolin analogue **2.28** (~1 mg) was dissolved in 20% piperidine/THF (0.5 mL) and the yellow solution stirred at rt for 15 min. The solution was diluted with H_2O (10 mL) and loaded onto a C_{18} cartridge. The cartridge was washed with H_2O, then the variolin material washed off with MeOH/DCM. To the yellow solution thus obtained was added a small amount of TFA, and the orange solution examined by HPLC.

Depreotection of variolin analogue 2.28



Variolin analogue **2.28** (~1 mg) was dissolved in triflic acid (200 μL) and the deep red solution stirred at rt for 2 h. The solution was diluted with H_2O (5.0 mL) and loaded onto a C_{18} cartridge. The cartridge was washed with H_2O, and the variolin material washed off with MeOH/DCM. The yellow solution thus obtained was examined by HPLC.

Deoxyvariolin B (DVB)



DVB was synthesised via the slightly modified procedure of Anderson and Morris.[87] **2.26** (180 mg, 0.35 mmol) was dissolved in triflic acid (2 mL) and the deep orange/red solution stirred at rt for 2.5 h. The solution was cooled to 0 °C and aq NH_3 was added dropwise until a yellow precipitate had formed and the solution was alkaline. Sat aq $NaHCO_3$ solution was added carefully until effervescing ceased, the suspension filtered and the yellow/green solid washed with H_2O. This crude material was dissolved in THF (40 mL), DIOL (1.5 g) added and the suspension slowly diluted with hexanes (60 mL) with vigorous swirling. The resulting yellow slurry was loaded onto a DIOL column (8.5g diol) and flash chromatography, eluting with 2% $MeOH/CH_2Cl_2$, afforded DVB as a yellow microcrystalline solid (84 mg, 88% yield).

Mp: dec at ~200-230 °C (lit. dec at ~220-240).[87]

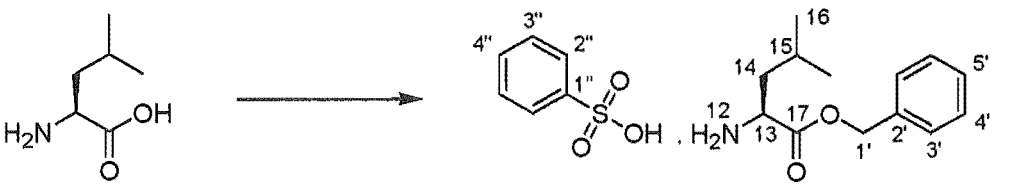
^{1}H NMR (500 MHz, $(CD_3)SO$): δ 6.55 (br s, 2H, 2'-NH_2), 7.06 (d, J = 5.4 Hz, 1H, H-5'), 7.08 (dd, J = 4.4, 7.8 Hz, 1H, H-3), 7.63 (d, J = 6.4 Hz, 1H, H-7), 7.68 (d, J = 6.8 Hz, 1H, H-6), 8.22

(d, J = 5.4 Hz, 1H, H-6'), 8.35-8.80 (br s, 1H, 9-N*H*), 8.45 (dd, J = 1.5, 4.9 Hz, 1H, H-2), 8.92 (dd, J = 1.5, 8.3 Hz, 1H, H-4), 9.20-9.55 (br s, 1H, 9-N*H*).

HRMS (ESI): Calcd for $C_{14}H_{12}N_7$ (MH^+) *m/z* = 278.1154, found *m/z* = 278.1153.

IC_{50} (P388): 0.47 μM

L-Leucine benzyl ester benzenesulfonate[138]



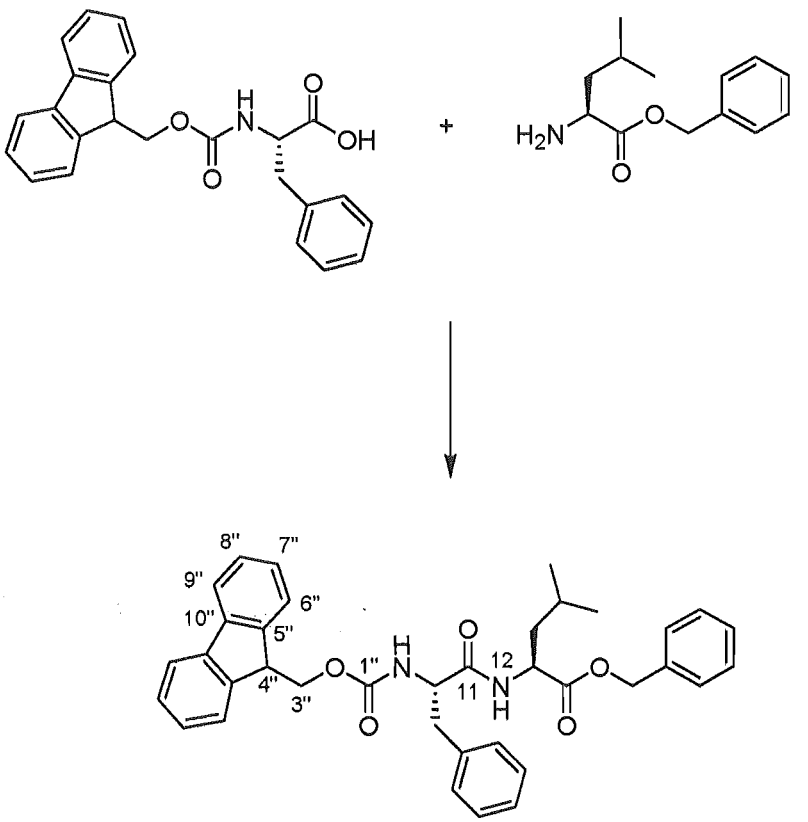
A solution of L-leucine (1.00 g, 7.62 mmol) and benzenesulfonic acid (1.33 g, 8.39 mmol) in benzyl alcohol (5.0 mL) was heated at 115 °C for 1 h at atmospheric pressure, then for a further 1.5 h under reduced pressure (10 mm Hg), with only a small amount of benzyl alcohol (<1 mL) being removed by distillation during this time. The solution was transferred to a mortar and pestle, allowed to cool to rt, then ground with diethyl ether (30 mL). The resulting precipitate was collected by filtration, washed with diethyl ether and dried *in vacuo* to afford the benzyl ester salt as a white crystalline solid (2.34 g, 81% yield).

Mp: 169-170 °C. [lit. 167-8 °C (ethanol/diethyl ether)].[141]

^{1}H NMR (500 MHz, d_6-DMSO): δ 0.86 (d, J = 4.9 Hz, 6H, H-16), 1.57-1.72 (m, 3H, H-14 and H-15), 4.05 (t, J = 7.1 Hz, 1H, H-13), 5.23 (s, 2H, H-1'), 7.31-7.42 (m, 8H, Ar), 7.61 (d, J = 7.7 Hz, 2H, H-2"), 8.36 (br s, 3H, H-12).

^{13}C NMR (75 MHz, d_6-DMSO): δ 22.2 (C-16), 23.9 (C-15), 39.1 (C-14), 50.8 (C-13), 67.4 (C-1'), 125.7 (C-2"), 127.9 (Ar), 128.47 (Ar), 128.67 (Ar), 128.74 (Ar), 128.81 (Ar), 123.3 (C-2'), 148.1 (C-1"), 170.0 (C-17).

_N_-(9-Fluorenylmethoxycarbonyl)-L-phenylalanyl-L-leucine benzyl ester



To a solution/suspension of L-leucine benzyl ester benzenesulfonate salt (100 mg, 264 μmol), Fmoc-L-phenylalanine (107 mg, 276 μmol), DCC (71 mg, 344 μmol) and HOBT (53 mg, 392 μmol) in THF (10 mL) was added TEA (55 μL, 397 μmol), and the white suspension stirred at rt for 18 h. This was then diluted with CH_2Cl_2 (10 mL), washed with sat brine solution (10 mL), filtered and concentrated *in vacuo*. To the crude material thus obtained was added CH_2Cl_2 (10 mL) and silica (1 g), and the resulting suspension stirred at rt for 5 min. Hexanes (10 mL) were next added with vigorous stirring, and the suspension loaded onto a column of silica (9 g) in hexanes. Flash chromatography, eluting with 10-15% EtOAc/hexanes, afforded the dipeptide as a white amorphous solid (140 mg, 95% pure, 85% yield).

Mp: 108-110 °C (lit. 154-5 °C).[95]

^{1}H NMR (500 MHz, d_6-DMSO): δ 0.82 (d, J = 6.4 Hz, 3H, H-16a), 0.88 (d, J = 6.4 Hz, 3H, H-16b), 1.51-1.68 (m, 3H, H-14 and H-15), 2.73 (dd, J= 11.2, 14.2 Hz, 1H, H-6a), 2.94 (dd, J = 3.4, 13.7 Hz, 1H, H-6b), 4.08-4.13 (m, 3H, H-3" and H-4"), 4.29 (m, 1H, H-5), 4.36 (m, 1H, H-13), 5.12 (s, 2H, H-1'), 7.17 (t, J = 7.3 Hz, 1H, H-10), 7.23-7.36 (m, 6H, H-3', H-4', H-8, H-5',

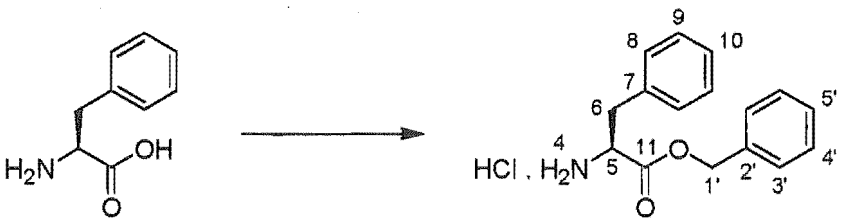
H-9, and H-7''), 7.49 (td, J = 3.2, 8.8 Hz, 2H, H-8''), 7.59-7.64 (m, 3H, H-4 and H-6''), 7.86 (d, J = 7.3 Hz, 2H, H-9''), 8.46 (d, J = 7.8 Hz, 1H, H-12).

^{13}C NMR (75 MHz, d_6-DMSO): δ 21.5 (C-16a), 22.9 (C-16b), 24.4 (C-15), 37.5 (C-6), 39.4 (C-14), 46.7 (C-4''), 50.7 (C-13), 56.0 (C-5), 65.8 (C-3''), 66.2 (C-1'), 120.3 (C-9''), 125.5 (C-6''), 126.5 (C-10), 127.3 (C-7''), 127.84 (C-8''), 128.03 (Ar), 128.22 (Ar), 128.27 (Ar), 128.6 (Ar), 129.4 (C-8), 136.1 (C-2'), 138.3 (C-7), 140.8 (C-10''), 143.9 (C-5''), 156.0 (C-1''), 172.19 (C-11), 172.41 (C-17).

IR (KBr): υ_{max} 3314, 3071, 2959, 1740, 1693, 1659, 1535, 1450, 1261, 1146, 1034, 741, 698.

HRMS (ESI): Calcd for $C_{37}H_{39}N_2O_5$ (MH^+) *m/z* = 591.2859, found *m/z* = 591.2863.

L-Phenylalanine benzyl ester hydrochloride



This was prepared from L-phenylalanine via the slightly modified procedure of Miller and Waelsch.[138] A solution of L-phenylalanine (1.00 g, 6.1 mmol) and benzenesulfonic acid (1.05 g, 6.7 mmol) in benzyl alcohol (12.0 mL) was heated to 120 °C under reduced pressure (10 mm Hg). When about half of the benzyl alcohol (~5 mL) had been removed by distillation the solution was transferred to a mortar and pestle, allowed to cool to rt, then ground with diethyl ether (50 mL). The resulting precipitate was collected by filtration, washed with diethyl ether and dried *in vacuo* to give white crystals (2.14 g). Examination of this material by ^{1}H NMR revealed that only ~50% esterification had occurred, so the material was again dissolved in benzyl alcohol (10 mL), benzenesulfonic acid (1.00 g, 6.32 mmol) added, and about half of the benzyl alcohol (~5 mL) removed by distillation under reduced pressure (10 mm Hg) at 120 °C over 3 h. The solution was then transferred to a mortar and pestle, allowed to cool to rt, and ground with diethyl ether (100 mL). The resulting precipitate was collected by filtration, washed with diethyl ether and dried *in vacuo* to afford the benzyl ester salt as white crystals (1.96 g). Examination of this material by ^{1}H NMR showed it to be of insufficient purity, and so it was decided to convert the benzyl ester to the hydrochloride salt for purification purposes.

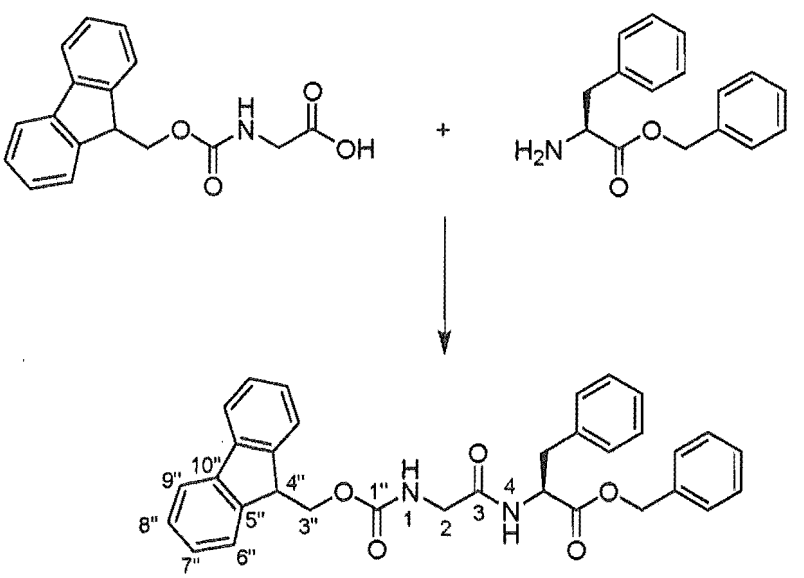
Thus, to a suspension of the benzenesulfonate salt in $CHCl_3$ (10 mL) was added TEA (1.30 mL, 9.4 mmol) and the white suspension stirred at rt for 10 min. Diethyl ether (40 mL) was added, and after 1 h at -20 °C the suspension was filtered and the filtrate collected and concentrated *in vacuo*. The crude material thus obtained was dissolved in THF (30 mL) and diluted with 1M aq HCl (20 mL). The resulting solution with cloudy precipitate was concentrated *in vacuo* to a volume of ~5 mL, diluted with MeOH (30 mL) and concentrated *in vacuo* to close to dryness, dissolved in MeOH (30 mL), concentrated *in vacuo* and left at reduced pressure (0.03 mm Hg) overnight. The crude material thus obtained was dissolved in warm benzyl alcohol (20 mL), diluted with diethyl ether (60 mL) and left at -20 °C for 1h. The resulting crystals were collected by filtration to afford the benzyl ester hydrochloride salt as white needles (342 mg, 19% yield). Addition of ether (100 mL) to the mother liquor, followed by cooling to -20 °C for 1h, afforded a further 120 mg (26% total yield) of the title compound in high purity.

Mp: 201-204 °C (lit. 207-9 °C).[142]

^{1}H NMR (500 MHz, d_6-DMSO): δ 3.09 (dd, J = 7.8, 14.2 Hz, 1H, H-6a), 3.22 (dd, J = 5.4, 13.7 Hz, 1H, H-6b), 4.32 (t, J = 6.6 Hz, 1H, H-5), 5.11 (d, J = 12.2 Hz, 1H, H-1'a), 5.15 (d, J = 12.2 Hz, 1H, H-1'b), 7.18-7.36 (m, 10H, Ar), 8.71 (br s, 3H, H-4).

^{13}C NMR (75 MHz, d_6-DMSO): δ 36.0 (C-6), 53.2 (C-5), 67.1 (C-1'), 127.3 (Ar), 128.37 (Ar), 128.42 (Ar), 128.45 (Ar), 128.65 (Ar), 125.5 (Ar), 134.6 (C-7), 134.8 (C-2'), 168.9 (C-11).

***N*-(9-Fluorenylmethoxycarbonyl)glycyl-L-phenylalanine benzyl ester**



To a solution of Fmoc-glycine (288 mg, 0.97 mmol), L-phenylalanine benzyl ester hydrochloride (283 mg, 0.97 mmol), EDCI (242 mg, 1.26 mmol) and HOBT (197 mg, 146 mmol) in THF (30mL) was added TEA (336 μL, 2.42 mmol), and the resulting white suspension stirred at rt for 18 h. This was then diluted with CH_2Cl_2 (30 mL), washed with 1.0 M aq HCl (50 mL), sat brine solution (50 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude residues thus obtained were purified by flash chromatography on silica, eluting with 1:1 EtOAc/hexanes, to afford the dipeptide as a white foam (454 mg, 95% pure, 83% yield).

Mp: 142-144 °C.

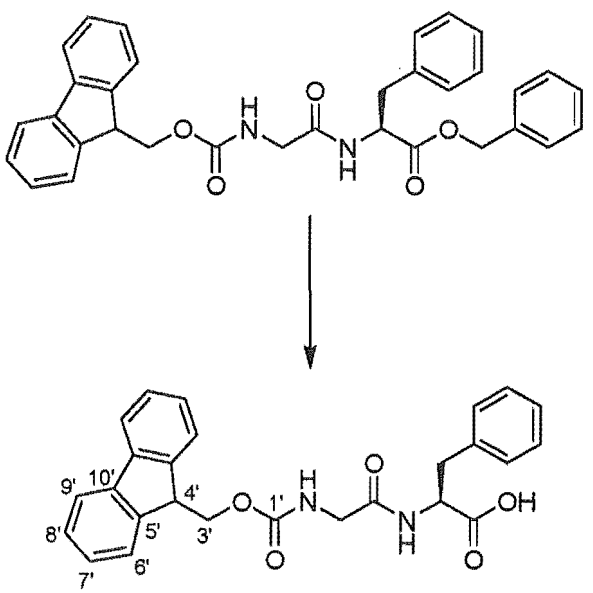
^{1}H NMR (500 MHz, d_6-DMSO): δ 2.94 (dd, J = 8.3, 13.7 Hz, 1H, H-6a), 3.03 (dd, J = 6.4, 13.7 Hz, 1H, H-6b), 3.59 (dd, J = 6.4, 17.1 Hz, 1H, H-2a), 3.64 (dd, J = 6.4, 17.1 Hz, 1H, H-2b), 4.21 (t, J = 6.8 Hz, 1H, H-4''), 4.27 (d, J = 6.8 Hz, 2H, H-3''), 4.53 (dd, J = 7.8, 14.2 Hz, 1H, H-5), 5.05 (d, J = 12.7 Hz, 1H, H-1'a), 5.09 (d, J = 12.7 Hz, 1H, H-1'b), 7.17-7.27 (m, 7H, H-7'', H-8, H-9 and H-10), 7.31-7.36 (m, 5H, H-3', H-4', H-5'), 7.41 (t, J = 7.6 Hz, 2H, H-8''), 7.51 (t, J = 6.4 Hz, 1H, H-1), 7.71 (d, J = 7.3 Hz, 2H, H-6''), 7.89 (d, J = 7.3 Hz, 2H, H-9''), 8.37 (d, J = 7.8 Hz, 1H, H-4).

^{13}C NMR (75 MHz, d_6-DMSO): δ 36.8 (C-6), 42.2 (C-2), 46.7 (C-4''), 53.8 (C-5), 65.9 (C-3''), 66.2 (C-1'), 120.2 (C-9''), 125.4 (C-6''), 126.7 (C-10), 127.2 (C-7''), 127.78 (C-8''), 127.96 (Ar), 128.16 (Ar), 128.43 (Ar), 128.52 (Ar), 129.2 (C-8), 135.8 (C-2'), 137.0 (C-7), 140.8 (C-10''), 144.0 (C-5''), 156.5 (C-1''), 169.4 (C-3), 171.4 (C-11).

IR (KBr): υ_{max} 3329, 3071, 2959, 1732, 1659, 1535, 1450, 1234, 1161, 1049, 741, 702.

HRMS (ESI): Calcd for $C_{33}H_{30}N_2O_5$ (MH^+) *m/z* = 535.2233, found *m/z* = 535.2215.

_N_-(9-Fluorenylmethoxycarbonyl)glycyl-L-phenylalanine



10% Pd/C (450 mg) was added to a solution of FmocGFOBn (448 mg, 95% pure, 0.80 mmol) in THF (50 mL), and the black suspension subjected to 3 succesive vacuum/H_2 cycles. This was stirred under H_2 for 18 h at rt, then filtered through a celite plug and concentrated *in vacuo* to give the dipeptide acid as a white foam (353 mg, 95% pure, 95% yield).

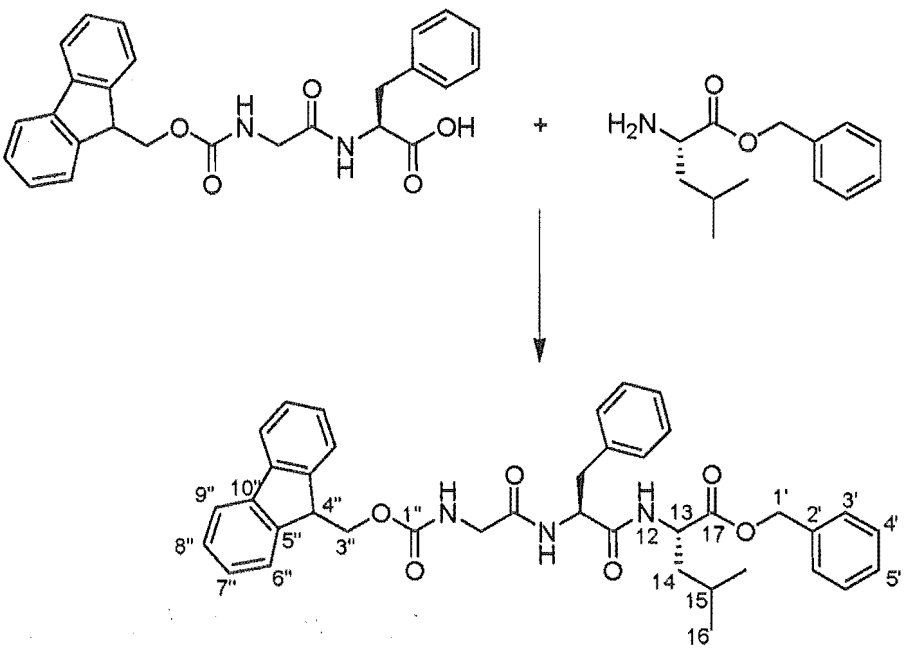
Mp: 187-189 °C (lit. 204-6 °C).[143]

^{1}H NMR (500 MHz, d_6-DMSO): δ 2.88 (dd, J = 8.8, 14.2 Hz, 1H, H-6a), 3.04 (dd, J = 5.4, 14.2 Hz, 1H, H-6b), 3.56 (dd, J = 6.4, 17.1 Hz, 1H, H-2a), 3.63 (dd, J = 6.4, 17.1 Hz, 1H, H-2b), 4.21 (t, J = 6.8 Hz, 1H, H-4'), 4.26 (d, J = 6.4 Hz, 2H, H-3'), 4.43 (td, J = 5.4, 8.3 Hz, 1H, H-5), 7.17-7.27 (m, 5H, H-8, H-9, H-10), 7.32 (t, J = 7.3 Hz, 2H, H-7'), 7.41 (t, J = 7.3 Hz, 2H, H-8'), 7.49 (t, J = 6.1 Hz, 1H, H-1), 7.70 (d, J = 7.3 Hz, 2H, H-6'), 7.89 (d, J = 7.8 Hz, 2H, H-9'), 8.12 (d, J = 7.8 Hz, 1H, H-4).**^{13}C NMR** (75 MHz, d_6-DMSO): δ 36.9 (C-6), 43.2 (C-2), 46.7 (C-4'), 53.6 (C-5), 65.9 (C-3'), 120.2 (C-9'), 125.4 (C-6'), 126.6 (C-10), 127.2 (C-7'), 127.8 (C-8'), 128.3 (C-9), 129.3 (C-8), 137.5 (C-7), 140.8 (C-10'), 144.0 (C-5'), 156.6 (C-1'), 169.1 (C-3), 172.9 (C-11).

IR (KBr): υ_{max} 3329, 1732, 1597, 1535, 1450, 1412, 1234, 1161, 1088, 1045, 995, 918, 760, 741, 702.

HRMS (ESI): Calcd for $C_{26}H_{25}N_2O_5$ (MH^+) *m/z* = 445.1763, found *m/z* = 445.174.

_N_-(9-Fluorenylmethoxycarbonyl)glycyl-L-phenylalanyl-L-leucine benzyl ester



To a solution of FmocGF (350 mg, 95% pure, 0.75 mmol), L-leucine benzyl ester benzenesulfonate (329 mg, 0.87 mmol), EDCI (196 mg, 1.02 mmol) and HOBT (160 mg, 1.18 mmol) in THF (50 mL) was added TEA (273 μL, 1.97 mmol), and the resulting white suspension stirred at rt for 18 h. This was then diluted with CH_2Cl_2 (30 mL), washed with 1.0 M aq HCl (30 mL), sat brine solution (40 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude residues thus obtained were purified by flash chromatography on silica, eluting with 30% EtOAc/CH_2Cl_2, to afford the tripeptide as a clear colourless glass (354 mg, 73% yield).

Mp: 125-128 °C [lit. 130-2 °C (3:1 *n*-hexane/EtOAc)].[144]

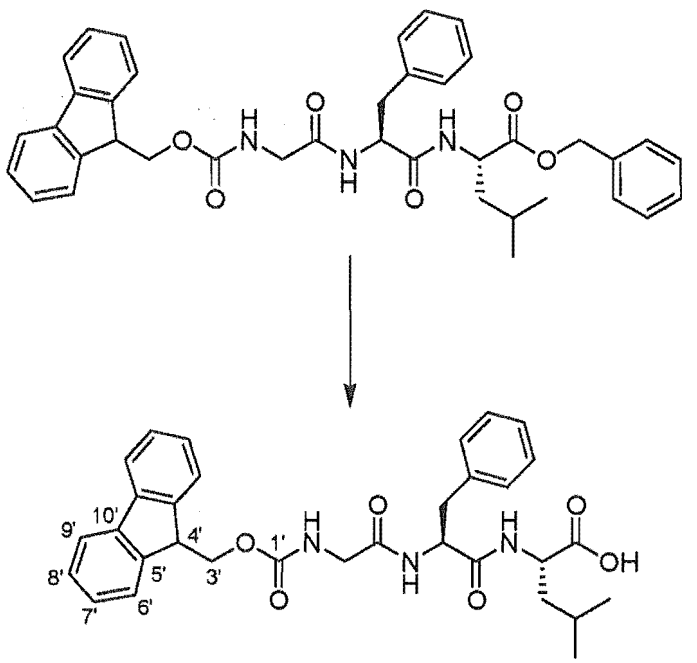
^{1}H NMR (500 MHz, d_6-DMSO): δ 0.82 (d, J = 5.9 Hz, 3H, H-16a), 0.88 (d, J = 6.4 Hz, 3H, H-16b), 1.49-1.66 (m, 3H, H-14 and H-15), 3.49 (dd, J = 5.9, 16.6 Hz, 1H, H-2a), 3.61 (d, J = 6.4, 17.1 Hz, 1H, H-2b), 4.20 (t, J = 6.8 Hz, 1H, H-4"), 4.24 (d, J = 5.9 Hz, 2H, H-3"), 4.33 (m, 1H, H-13), 4.55 (td, J = 3.9, 9.3 Hz, 1H, H-5), 5.11 (s, 2H, H-1'), 7.19-7.21 (m, 5H, H-8, H-9, H-10), 7.31 (t, J = 7.8 Hz, 2H, H-7"), 7.34-7.36 (m, 5H, H-3', H-4', H-5'), 7.50 (t, J = 7.3 Hz, 2H, H-8'), 7.48 (t, J = 6.1 Hz, 1H, H-1), 7.69 (d, J = 7.8 Hz, 2H, H-6"), 7.88 (d, J = 7.8 Hz, 2H, H-9"), 8.04 (d, J = 8.3 Hz, 1H, H-4), 8.46 (d, J = 7.3 Hz, 1H, H-12).

^{13}C NMR (75 MHz, d_6-DMSO): δ 21.5 (C-16a), 22.8 (C-16b), 24.3 (C-15), 38.1 (C-6), 39.9 (C-13), 43.2 (C-2), 46.7 (C-4''), 50.6 (C-14), 53.6 (C-5), 65.9 (C-3''), 66.1 (C-1'), 120.2 (C-9''), 125.4 (C-6''), 126.4 (C-10), 127.2 (C-7''), 127.77 (C-8''), 127.97 (Ar), 128.14 (Ar), 128.20 (C-9), 128.55 (C-3'), 129.3 (C-8), 136.0 (C-2'), 137.8 (C-7), 140.8 (C-10''), 143.9 (C-5''), 156.6 (C-1''), 168.9 (C-3), 171.5 (C-11), 172.2 (C-17).

IR (KBr): υ_{max} 3306, 3071, 2959, 1728, 1659, 1528, 1450, 1250, 1153, 1049, 741, 698.

HRMS (ESI): Calcd for $C_{39}H_{42}N_3O_6$ (MH^+) *m/z* = 648.3074, found *m/z* = 648.3048.

_N_-(9-Fluorenylmethoxycarbonyl)glycyl-L-phenylalanyl-L-leucine



10% Pd/C (350 mg) was added to a solution of FmocGFLOBn (350 mg, 0.54 mmol) in THF (60 mL), and the black suspension was subjected to 3 successive vacuum/H_2 cycles. This was stirred, under H_2, at rt for 18 h, then filtered through a celite plug and concentrated *in vacuo*. The crude residues thus obtained were purified by flash chromatography, eluting with 1:1 EtOAc/CH_2Cl_2 (2-20% MeOH), to give the tripeptide acid as a white foam (253 mg, 95% pure, 80% yield).

Mp: 94-97 °C.

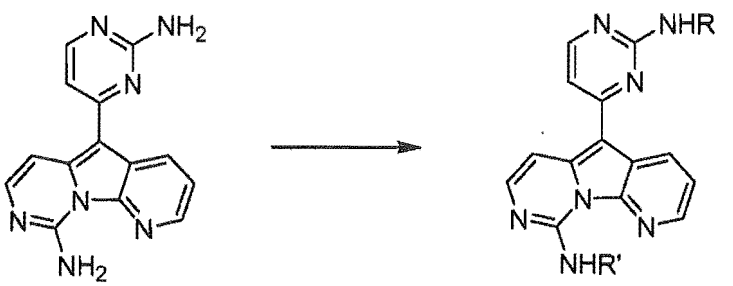
^{1}H NMR (500 MHz, d_6-DMSO): δ 0.83 (d, J = 6.8 Hz, 3H, H-16a), 0.88 (d, J = 6.4 Hz, 3H, H-16b), 1.52 (t, J = 7.1 Hz, 2H, H-14), 1.62 (m, 1H, H-15), 2.75 (dd, J = 9.8, 13.7 Hz, 1H, H-6a), 3.03 (dd, J = 3.9, 13.7 Hz, 1H, H-6b), 3.49 (dd, J = 16.6, 6.4 Hz, 1H, H-2a), 3.62 (dd, J = 5.9, 16.6 Hz, 1H, H-2b), 4.16-4.21 (m, 2H, H-4' and H-13), 4.24 (d, J = 6.8 Hz, 2H, H-3'), 4.55 (td, J = 3.9, 9.3 Hz, 1H, H-5), 7.14-7.17 (m, 1H, H-10), 7.19-7.24 (m, 4H, H-8 and H-9), 7.32 (t, J = 7.6 Hz, 2H, H-7'), 7.41 (t, J = 7.3 Hz, 2H, H-8'), 7.50 (t, J = 6.1 Hz, 1H, H-1), 7.69 (d, J = 7.3 Hz, 2H, H-6'), 7.88 (d, J = 7.8 Hz, 2H, H-9'), 8.00 (d, J = 8.3 Hz, 1H, H-4), 8.18 (d, J = 6.4 Hz, 1H, H-12).

^{13}C NMR (75 MHz, d_6-DMSO): δ 21.5 (C-16a), 23.0 (C-16b), 24.4 (C-15), 37.6 (C-6), 39.9 (C-14), 43.3 (C-2), 46.7 (C-4'), 50.5 (C-13), 53.6 (C-5), 65.9 (C-3'), 120.2 (C-9'), 125.4 (C-6'), 126.4 (C-10), 127.3 (C-7'), 127.8 (C-8'), 128.2 (C-9), 129.4 (C-8), 137.8 (C-7), 140.9 (C-10'), 144.0 (C-5'), 156.6 (C-1'), 168.9 (C-3), 171.3 (C-11), 174.1 (C-17).

IR (KBr): υ_{max} 3306, 3071, 2959, 1729, 1659, 1450, 1254, 1157, 1049, 995, 741, 702.

HRMS (ESI): Calcd for $C_{32}H_{36}N_3O_6$ (MH^+) *m/z* = 558.2604, found *m/z* = 558.2624.

9-amino-5-[2-{*N*-(9-fluorenylmethoxycarbonyl)glycyl}pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 2.30



R = H or Fmocglycyl
R' = H or Fmocglycyl
2.30: R = Fmocglycyl, R' = H

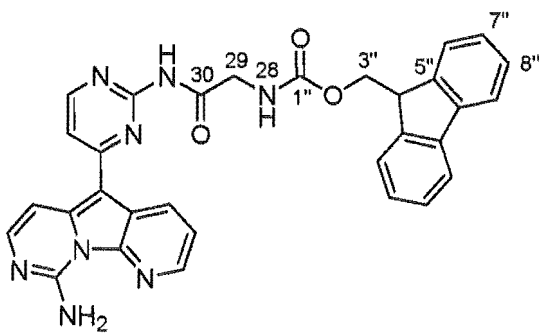
a) To a mixture of DVB (10.0 mg, 36 μmol) and Fmoc-glycine acid chloride (11.4 mg, 36 μmol) was simultaneously added THF (5 mL) and TEA (5.5 μL, 40 μmol) with stirring, and the resulting yellow solution was stirred at rt for 18 h. At this time the reaction solution was examined by HPLC using the standard gradient elution protocol.

b) A solution of DVB (10.0 mg, 36 μmol), Fmoc-glycine (12.5 mg, 40 μmol), EDCI (9.0 mg, 47 μmol) and HOBT (7.3 mg, 54 μmol) in pyridine (1 mL) was stirred at rt for 19 h. At this time the solution was analysed by HPLC using the standard gradient elution protocol. After a further 24 h stirring at rt the solution was again analysed by HPLC.

c) A solution of DVB (10.0 mg, 36 μmol), Fmoc-glycine (10.7 mg, 36 μmol) and HBTU (15.0 mg, 40 μmol) in DMF (0.5 mL) was stirred at rt for 20 h. At this time the solution was analysed by HPLC using the standard gradient elution protocol.

d) To a solution of DVB (10.0 mg, 36 μmol) and TEA (25 μL, 180 μmol) in pyridine (1 mL) at -10 °C was added a solution of Fmoc-glycine acid chloride (34 mg, 108 μmol) in THF (1.0 mL) dropwise over 1 min, and the yellow solution was allowed to warm slowly to rt over 16 h. The solutions was analysed by HPLC. Dry MeOH (100 μL) was added and the solution stirred at rt for 24 h, then the solution again analysed by HPLC using the standard gradient elution protocol. To the solution was added a slurry of neutralised silica (~1 g) in CH_2Cl_2 (3 mL), and, with vigorous swirling, the resulting slurry was slowly diluted with hexanes (20 mL). The resulting yellow/brown slurry was loaded onto a column of neutralised silica (9 g) in CH_2Cl_2, and the variolin material eluted with 2-5% MeOH/CH_2Cl_2 (0.1% TEA). The yellow variolin-containing fractions (as determined by tlc) were combined, concentrated *in vacuo*, and examined by HPLC.

e) A solution of DVB (16.0 mg, 58 μmol), Fmoc-glycine acid chloride (54.7 mg, 173 μmol) and DMAP (1.0 mg, 8 μmol) in pyridine (1 mL) was stirred at rt for 17 h. At this time the solution was diluted with CH_2Cl_2 (3 mL), DIOL (~1 g) added, and the slurry slowly diluted with hexanes (10 mL) with vigorous swirling to coat the diol. The resulting yellow slurry was loaded onto a column of DIOL (9 g) in CH_2Cl_2, and the variolin material eluted with 0.5-10% MeOH/CH_2Cl_2. Further purification of the yellow amorphous material thus obtained by repeated flash chromatography on neutralised silica, eluting with 2% MeOH/ CH_2Cl_2 (0.1% TEA), afforded the monoacetylated analogue **2.30** as a yellow amorphous solid (1.1 mg, 80% pure, 3% yield).



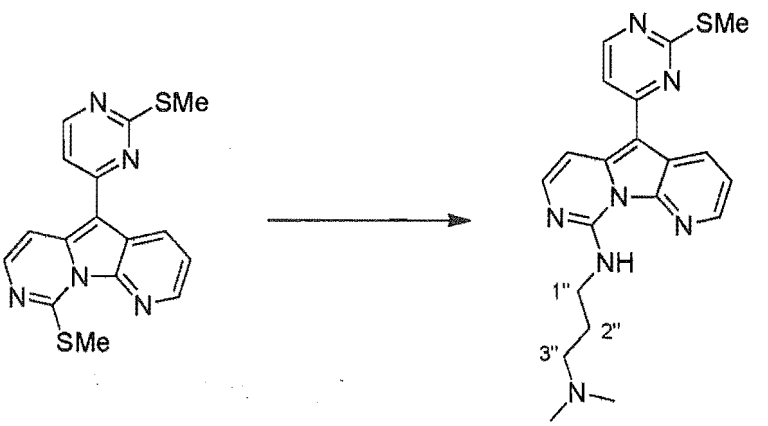
^{1}H NMR (500 MHz, d_6-DMSO): δ 4.03 (d, J = 5.9 Hz, 2H, H-29), 4.28 (t, J = 6.8 Hz, 1H, H-4"), 4.33 (d, J = 6.8 Hz, 2H, H-3"), 7.35 (t, J = 7.3 Hz, 2H, H-7"), 7.43 (t, J = 7.3 Hz, 2H, H-8"), 7.58 (dd, J = 4.4, 7.8 Hz, 1H, H-3), 7.61 (d, J = 5.4 Hz, 1H, H-5'), 7.66-7.68 (m, 2H, H-7 and H-28), 7.76 (d, J = 7.3 Hz, 2H, H-6"), 7.90 (d, J = 7.8 Hz, 2H, H-9"), 8.05 (d, J = 6.4 Hz, 1H, H-6), 8.4-8.8 (br, 1H, 9-N*H*a), 8.45 (dd, J = 1.2, 4.4 Hz, 1H, H-2), 8.55 (d, J = 5.9 Hz, 1H, H-6'), 9.19 (dd, J = 1.1, 7.8 Hz, 1H, H-4), 9.2-9.6 (br, 1H, 9-N*H*b), 10.63 (s, 1H, 2'-N*H*).

^{13}C NMR (75 MHz, d_6-DMSO): δ 45.8 (C-29), 46.8 (C-4"), 66.1 (C-3"), 98.8 (C-5), 102.0 (C-6), 112.8 (C-5'), 120.3 (C-9"), 121.0 (C-3), 121.5 (C-4a), 125.4 (C-6"), 127.2 (C-7"), 127.8 (C-8"), 129.9 (C-4), 139.5 (C-5a), 140.3 (C-2), 140.9 (C-10"), 143.1 (C-10a), 144.0 (C-5"), 145.7 (C-7), 149.8 (C-9), 157.5 (C-2'), 156.8 (C-1"), 157.7 (C-6'), 161.8 (C-4'), 168.9 (C-30).

HRMS (ESI): Calcd for $C_{31}H_{25}N_8O_3$ (MH^+) *m/z* = 557.2050, found *m/z* = 557.2060.

6.3 Experiments described in Chapter 3

9-[3-(Dimethylamino)propylamino]-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.12



A solution of core structure **2.6** (12.0 mg, 35 μM) and *N*,*N*-dimethylaminopropaneamine (55 μL, 443 μM) in toluene (0.5 mL) was heated at 100 °C for 24 h. The yellow solution was allowed to cool to rt then concentrated *in vacuo* to leave an oily yellow residue. The crude material thus obtained was purified by flash chromatography on C_{18}, eluting with 50-70% MeOH/H_2O (0.1% TFA). The orange material thus obtained was dissolved in MeOH (0.7 mL), diluted with CH_2Cl_2 (10 mL), stirred with solid K_2CO_3 (during which time the solution lightened from an orange to a yellow colour), filtered and concentrated *in vacuo* to give the title compound as a dark yellow/green gum (11.6 mg, 98% pure, 98% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.98 (p, J = 6.8 Hz, 2H, H-2"), 2.30 (s, 6H, N(CH_3)$_2$), 2.49 (t, J = 7.1 Hz, 2H, H-3"), 2.68 (s, 3H, SCH_3), 3.81 (m, 2H, H-1"), 7.31 (d, J = 5.4 Hz, 1H, H-5'), 7.46 (dd, J = 4.9, 8.3 Hz, 1H, H-3), 7.48 (d, J = 6.8 Hz, 1H, H-6), 7.73 (d, J = 6.3 Hz, 1H, H-7), 8.36 (dd, J = 1.5, 4.9 Hz, 1H, H-2), 8.64 (d, J = 5.4 Hz, 1H, H-6'), 8.71 (dd, J = 1.5, 8.3 Hz, 1H, H-4), 10.22 (t, J = 5.4 Hz, 1H, 9-N*H*).

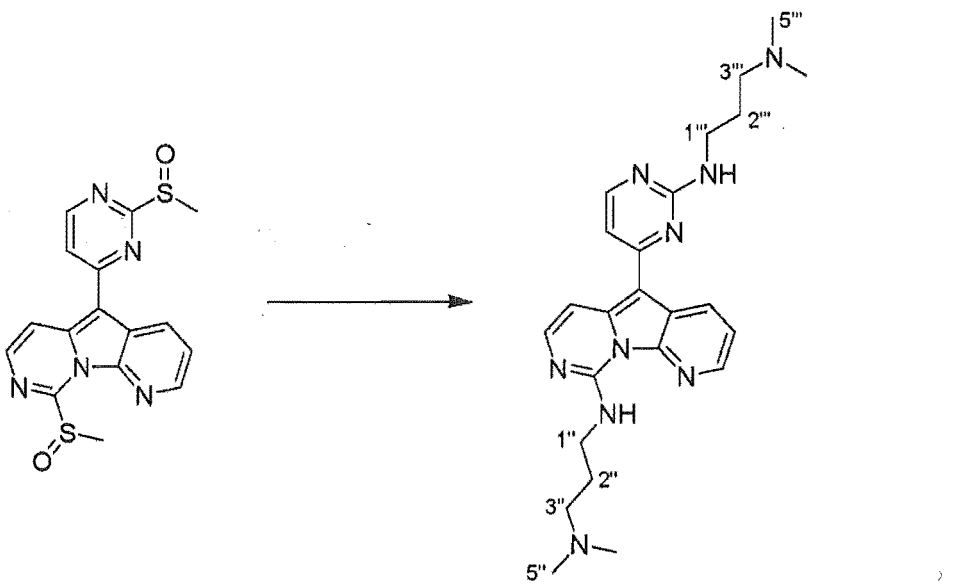
^{13}C NMR (75 MHz, $CDCl_3$): δ 14.3 (2'-SCH_3), 27.14 (C-2"), 39.4 (C-1"), 45.6 (C-5"), 57.2 (C-3"), 99.5 (C-5), 100.8 (C-6), 112.4 (C-5'), 120.3 (C-3), 121.8 (C-4a), 128.2 (C-4), 139.3 (C-5a), 139.6 (C-2), 143.6 (C-10a), 144.1 (C-7), 149.2 (C-9), 156.2 (C-6'), 161.4 (C-4'), 172.2 (C-2').

IR (KBr): υ_{max} 2950-2750 (series of weak bands), 1686, 1558, 1473, 1458, 1404, 1285, 1207, 1184, 1130.

HRMS (ESI): Calcd for $C_{20}H_{24}N_7{}^{32}S$ (MH^+) *m/z* = 394.1814, found *m/z* = 394.1810.

IC_{50} (P388): 11 μM

9-[3-(Dimethylamino)propylamino]-5-[2-(3-(dimethylamino)propylamino)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.13



Oxidised core structure **2.25** (10.9 mg, 29 μmol, assuming quantitative yield in prior oxidation reaction) was heated with *N*,*N*-dimethylaminopropaneamine (56 μL, 443 μmol) in toluene (0.2 mL) at 105 °C for 19 h. The solution was allowed to cool to rt then concentrated *in vacuo*. The crude material thus obtained was purified by repeated flash chromatography on C_{18}, eluting with 30-60% MeOH/H_2O (0.1% TFA). The orange material thus obtained was dissolved in MeOH (0.5 mL), diluted with CH_2Cl_2 (10 mL), stirred with solid K_2CO_3 (during which time the solution lightened from an orange to a yellow colour), filtered and concentrated *in vacuo* to give the title compound as a thick yellow oil (8.3 mg, 63% yield over 2 steps, ie, from core structure **2.6**).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.87 (p, J = 6.8 Hz, 2H, H-2'''), 1.99 (p, J = 6.8 Hz, 2H, H-2''), 2.28 (s, 6H, H-5'''), 2.30 (s, 6H, H-5''), 2.46 (t, J = 7.3 Hz, 2H, H-3'''), 2.49 (t, J = 7.3 Hz, 2H,

H-3''), 3.61 (m, 2H, H-1'''), 3.80 (m, 2H, H-1''), 5.75 (br, 1H, 2'-N*H*), 6.93 (d, J = 5.4 Hz, 1H, H-5'), 7.42 (dd, J = 4.9, 8.3 Hz, 1H, H-3), 7.48 (d, J = 6.3 Hz, 1H, H-6), 7.65 (d, J = 6.3 Hz, 1H, H-7), 8.28 (d, J = 5.4 Hz, 1H, H-6'), 8.33 (dd, J = 1.5, 4.9 Hz, 1H, H-2), 8.70 (dd, J = 1.5, 7.8 Hz, 1H, H-4), 10.17 (t, J = 5.4, 1H Hz, 9-N*H*).

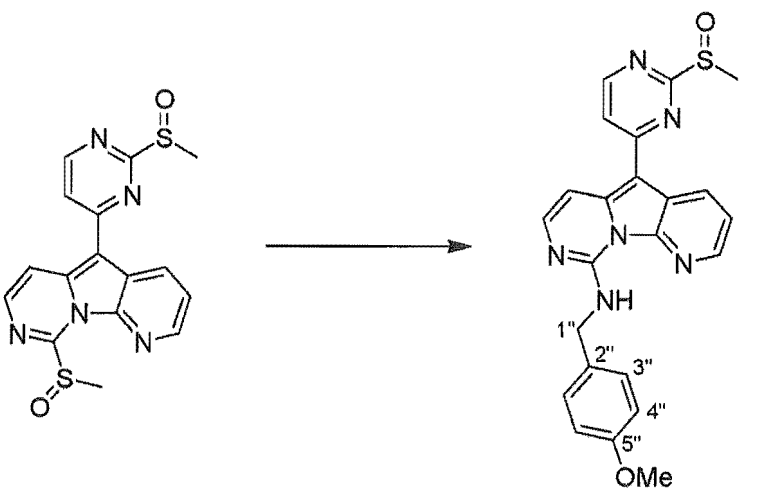
^{13}C NMR (75 MHz, $CDCl_3$): δ 27.17 (C-2''), 27.19 (C-2'''), 39.3 (C-1''), 40.6 (C-1'''), 45.52 (C-5''), 45.54 (C-5'''), 57.2 (C-3''), 57.9 (C-3'''), 100.6 (C-5), 101.1 (C-6), 107.5 (C-5'), 120.0 (C-3), 122.0 (C-4a), 128.3 (C-4), 138.5 (C-5a), 139.4 (C-2), 143.0 (C-7), 143.5 (C-10a), 149.2 (C-9), 157.5 (C-4'), 162.1 (C-2' or C-6'), 162.5 (C-2' or C-6').

IR ($CDCl_3$): υ_{max} 3449, 3252, 2950-2770 (series of weak bands), 1620-1470 (series of bands), 1261, 1188.

HRMS (ESI): Calcd for $C_{24}H_{34}N_9$ (MH^+) *m/z* = 448.2937, found *m/z* = 448.2945.

IC_{50} (P388): 6.0 μM

9-(4-Methoxybenzylamino)-5-[2-(methylsulfinyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.18



To a solution of oxidised core structure **2.25** (20 mg, 54 μmol, assuming quantitative yield in prior oxidation reaction) in CH_2Cl_2 (5.0 mL) was added *p*-methoxybenzylamine (8.0 μL, 61 μmol), and the solution stirred at rt for 10 min. The solvent was removed *in vacuo* and the yellow oil left under reduced pressure (0.03 mm Hg) for 48 h. The crude material thus obtained was purified by repeated flash chromatography on neutralised silica, eluting with 0.3-1%

MeOH/DCM (1% TEA), to give the monosubstituted sulfoxide as a thick yellow oil (16.6 mg, 63% yield over two steps, ie, from core structure **2.6**).

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.02 (s, 3H, 2'-SOC*H*$_3$), 3.82 (s, 3H, PMB OC*H*$_3$), 4.91 (d, J = 5.9 Hz, 2H, H-1''), 6.93 (d, J = 8.3 Hz, 2H, PMB H-4''), 7.42 (d, J = 8.8 Hz, 2H, PMB H-3''), 7.47 (dd, J = 4.6, 8.1 Hz, 1H, H-3), 7.61 (d, J = 6.9 Hz, 1H, H-6), 7.62 (d, J = 5.3 Hz, 2H, H-5'), 7.85 (d, J = 6.3 Hz, 1H, H-7), 8.32 (dd, J = 1.0, 3.9 Hz, 1H, H-2), 8.71 (d, J = 5.9 Hz, 1H, H-6'), 8.82 (dd, J = 1.5, 7.8 Hz, 1H, H-4), 10.47 (t, J = 5.4 Hz, 1H, 9-N*H*).

^{13}C NMR (75 MHz, $CDCl_3$): δ 40.2 (2'-SOCH_3), 44.4 (C-1''), 55.3 (OCH_3), 98.8 (C-5), 101.3 (C-6), 114.0 (PMB C-4''), 116.3 (C-5'), 120.8 (C-3), 121.6 (C-4a), 128.8 (C-4), 128.9 (C-3''), 129.9 (C-2''), 140.1 (C-2), 140.4 (C-5a), 143.7 (C-10a), 145.6 (C-7), 149.1 (C-9), 157.0 (C-6'), 159.0 (C-5''), 162.5 (C-4'), 173.3 (C-2').

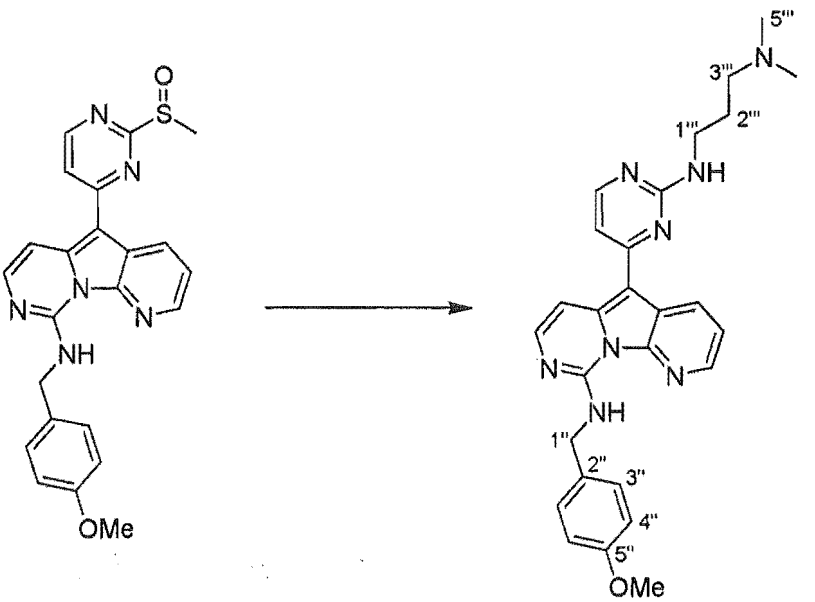
IR (KBr): υ_{max} 3060-2800 (series of weak bands), 1558, 1474, 1354, 1250, 1184, 1069, 1033, 980.

HRMS (ESI): Calcd for $C_{23}H_{21}N_6O_2{}^{32}S$ (MH^+) *m/z* = 445.1447, found *m/z* = 445.1445.

IC_{50} (P388): >28 μM (may have decomposed *in situ*).

NB - In subsequent reactions, described below, this intermediate was not purified; that is, it was generated and used *in situ*.

9-(4-Methoxybenzylamino)-5-[2-(3-(dimethylamino)propylamino)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.19



Monosulfoxide variolin **3.18** (26 mg, 59 μmol, assuming quantitative yield in prior oxidation reaction) was heated with *N,N*-dimethylaminopropaneamine (200 μL, 1.59 mmol) in toluene (0.8 mL) at 100 °C for24 h. The solution was allowed to cool to rt then concentrated *in vacuo* to leave a yellow oil, which was purified by repeated flash chromatography on C_{18}, eluting with 60% MeOH/H_2O (0.1% TFA). The orange oil thus obtained was dissolved in MeOH (0.5 mL), diluted with CH_2Cl_2 (10 mL), stirred with solid K_2CO_3 (during which time the solution lightened from an orange to a yellow colour), filtered and concentrated *in vacuo* to give the title compound as a yellow oil (19.7 mg, 98% pure, 68% yield over three steps, ie, from core structure **2.6**).

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.04 (p, J = 6.8 Hz, 2H, H-2'''), 2.56 (s, 6H, H-5'''), 2.86 (t, J = 7.3 Hz, 2H, H-3'''), 3.62 (br, 2H, H-1'''), 3.81 (s, 3H, OC*H*$_3$), 4.89 (d, J = 5.4 Hz, 2H, H-1''), 5.93 (br, 1H, 2'-N*H*), 6.91 (d, J = 8.8 Hz, 2H, H-4''), 6.94 (d, J = 5.4 Hz, 1H, H-5'), 7.38-7.43 (m, 3H, H-3 and H-3''), 7.47 (d, J = 6.8 Hz, 1H, H-6), 7.68 (d, J = 6.3 Hz, 1H, H-7), 8.25 (d, J = 5.4 Hz, 1H, H-6'), 8.27 (dd, J = 1.5, 4.9 Hz, 1H, H-2), 8.66 (dd, J = 1.5, 8.3 Hz, 1H, H-4), 10.37 (t, J = 5.6, 1H, 9-N*H*).

^{13}C NMR (75 MHz, $CDCl_3$): δ 25.8 (C-2'''), 39.3 (C-1'''), 44.0 (C-5'''), 44.4 (C-1''), 55.3 (O*C*H$_3$), 56.6 (C-3'''), 100.5 (C-5), 101.5 (C-6), 107.7 (C-5'), 114.1 (C-4''), 120.1 (C-3), 121.9 (C-4a),

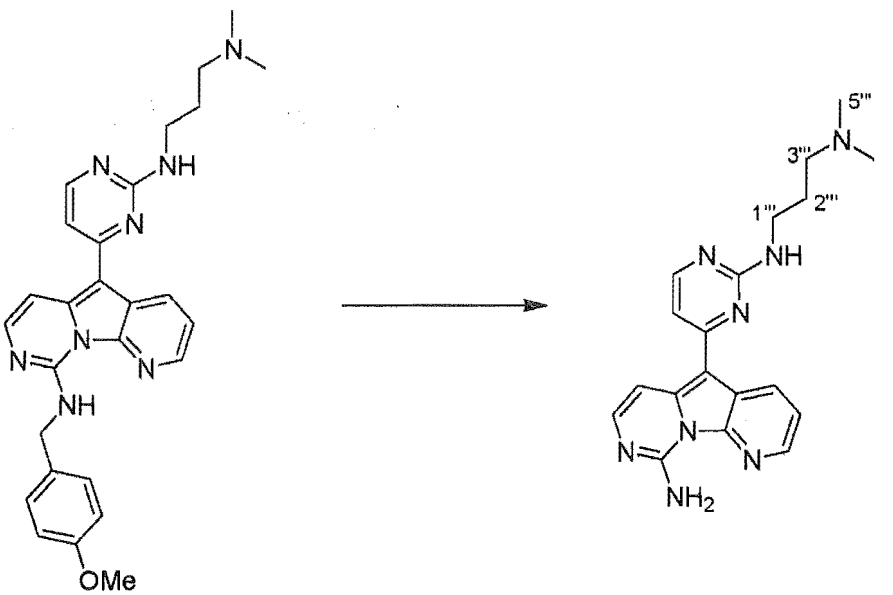
128.4 (C-4), 128.8 (C-3") 130.2 (C-2"), 138.6 (C-5a), 139.6 (C-2), 143.3 (C-7), 143.4 (C-10a), 149.1 (C-9), 156.9 (C-6'), 158.9 (C-5"), 162.07 (C-2'), 162.25 (C-4').

IR (KBr): υ_{max} 2950-2830 (series of weak bands), 1686, 1616, 1570, 1514, 1477, 1421, 1360, 1248, 1180, 1132, 1034, 808, 768.

HRMS (ESI): Calcd for $C_{27}H_{31}N_8O$ (MH^+) *m/z* = 483.2621, found *m/z* = 483.2623.

IC_{50} (P388): 2.1 μM

9-Amino-5-[2-(3-(dimethylamino)propylamino)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.15



PMB-protected variolin analogue **3.19** (13.0 mg, 27 μmol) was dissolved in triflic acid (0.3 mL) and the deep red/orange solution stirred at rt for 1h. The solution was cooled to 0 °C and aq NH_3 added dropwise to give a yellow precipitate and an alkaline solution. This suspension was loaded onto a C_{18} cartridge, and the product eluted with 40-80% MeOH/H_2O (0.1% TFA). The orange oil thus obtained was dissolved in MeOH (0.5 mL), diluted with CH_2Cl_2 (10 mL), stirred with solid K_2CO_3 (during which time the solution lightened from an orange to a yellow colour), filtered and concentrated *in vacuo* to give the title compound as a yellow oil (3.2 mg, 33% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.93 (p, J = 6.8 Hz, 2H, H-2'''), 2.37 (s, 6H, H-5'''), 2.59 (t, J = 6.8 Hz, 2H, H-3'''), 3.62 (m, 2H, H-1'''), 5.83 (br, 1H, 2'-N*H*), 5.0-7.6 (br, 1H, 9-N*H*) 6.95 (d, J = 5.4 Hz, 1H, H-5'), 7.46 (dd, J = 4.9, 8.3 Hz, 1H, H-3), 7.56 (d, J = 6.3 Hz, 1H, H-6), 7.62 (d, J = 6.8 Hz, 1H, H-7), 8.30 (d, J = 4.9 Hz, 1H, H-6'), 8.37 (dd, J = 1.2, 4.6 Hz, 1H, H-2), 8.72 (dd, J = 1.2, 8.1 Hz, 1H, H-4), 9.2-10.3 (br, 1H, 9-N*H*).

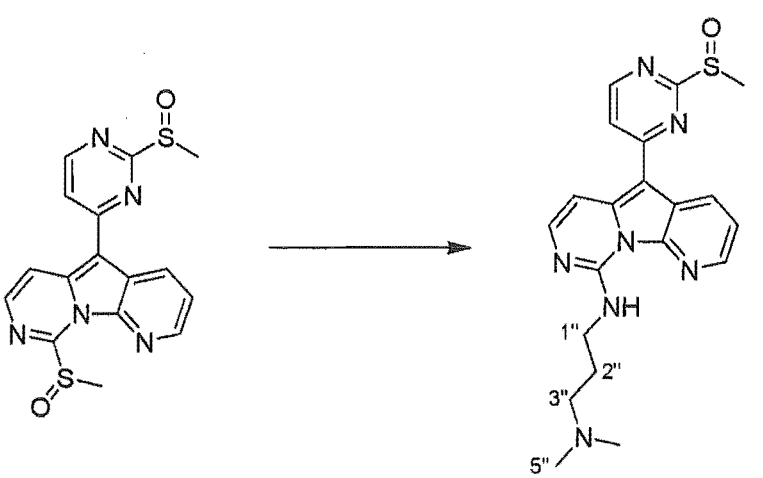
^{13}C NMR (75 MHz, $CDCl_3$): δ 26.8 (C-2'''), 40.3 (C-1'''), 45.1 (C-5'''), 57.6 (C-3'''), 101.1 (C-5), 102.5 (C-6), 107.7 (C-5'), 120.5 (C-3), 122.2 (C-4a), 128.7 (C-4), 138.0 (C-5a), 140.0 (C-2), 142.7 (C-7), 143.3 (C-10a), 149.6 (C-9), 157.6 (C-6'), 162.0 (C-4'), 162.5 (C-2').

IR (KBr): υ_{max} 3256-2750 (series of weak bands), 1560, 1508, 1491, 1271, 1205, 1124, 1011.

HRMS (ESI): Calcd for $C_{19}H_{23}N_8$ (MH^+) *m/z* = 363.2046, found *m/z* = 363.2042.

IC_{50} (P388): 2.5 μM.

9-[3-(Dimethylamino)propylamino]-5-[2-(methylsulfinyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.16



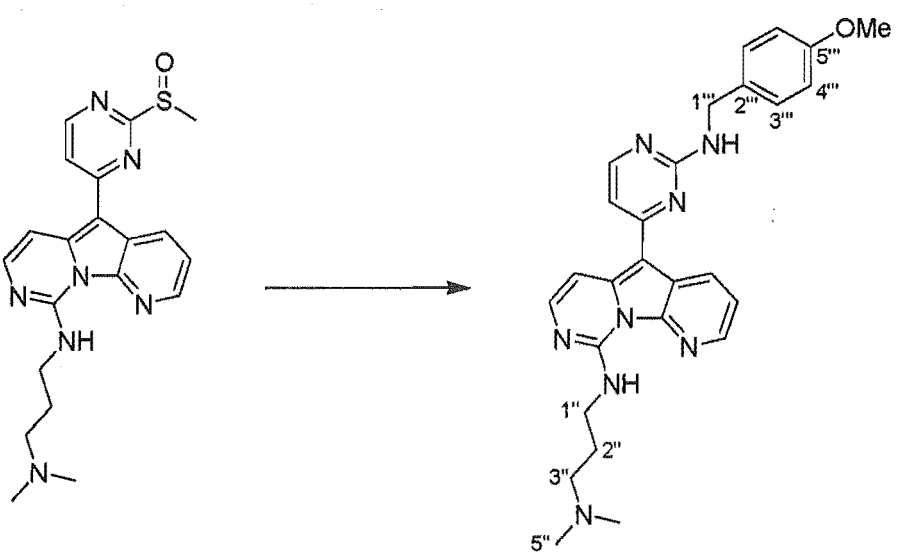
A solution of bis-sulfoxide variolin **2.25** (5.5 mg, 15 μmol, assuming quantitative yield in prior oxidation reaction) and *N,N*-dimethylpropylenediamine (4.0 μL, 31 μmol) in $CDCl_3$ was stirred at rt for 10 min. The solvent was removed *in vacuo* to leave a yellow oil, which was purified by repeated flash chromatography on C_{18}, eluting with 60-80% MeOH/H_2O (1% TFA), to give the TFA salt of the monosubstituted sulfoxide as an orange gum (yield not taken).

^{1}H NMR (500 MHz, CD_3OD): δ 2.25-2.31 (m, 2H, H-2''), 2.97 (s, 6H, H-5''), 2.99 (s, 3H, 2'-SOC*H*$_3$), 3.35 (t, J = 7.8 Hz, 2H, H-3''), 3.84 (td, J = 1.5, 6.3 Hz, 2H, H-1''), 7.45 (d, J = 5.4 Hz, 1H, H-5'), 7.55 (dd, J = 4.6, 8.1 Hz, 1H, H-3), 7.61 (d, J = 6.3 Hz, 1H, H-7), 7.70 (d, J = 6.8 Hz, 1H, H-6), 8.44 (dd, J = 1.2, 4.6 Hz, 1H, H-2), 8.54-8.60 (br, 1H, H-6'), 8.67 (dd, J = 1.2, 8.1 Hz, 1H, H-4).

IC_{50} (P388): >28 μM (may have decomposed *in situ*).

NB - In the subsequent reaction, described below, this intermediate was not purified; that is, it was generated and used *in situ*.

9-[3-(Dimethylamino)propylamino]-5-[2-(4-methoxybenzylamino)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.17



Monosulfoxide variolin **3.16** (24.0 mg, 59 μmol, assuming quantitative yields in prior oxidation and substitution reactions) was heated with *p*-methoxybenzylamine (100 μL, 765 μmol) in toluene (0.8 mL) at 105 °C for 22 h. The mixture was allowed to cool to rt then concentrated *in vacuo*. The crude material thus obtained was subjected to flash chromatography on C_{18}, eluting with 55-100% MeOH/H_2O (0.1% TFA). The orange material thus obtained was dissolved in MeOH (1.0 mL), diluted with CH_2Cl_2 (20 mL), stirred with solid K_2CO_3 (during which time the solution lightened from an orange to a yellow colour), filtered and concentrated *in vacuo*. This material was then purified by flash chromatography on C_{18}, eluting with 30-100%

CH_2Cl_2/MeOH, to give the title compound as a thick yellow oil (17.8 mg, 63% yield over three steps, ie, from core structure **2.6**).

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.00 (p, J = 6.8 Hz, 2H, H-2''), 2.33 (s, 6H, H-5''), 2.53 (t, J = 7.3 Hz, 2H, H-3''), 3.78-3.81 (m, 5H, H-1'' and OCH_3), 4.69 (d, J = 5.4 Hz, 2H, H-1'''), 5.50 (br, 1H, 2'-N*H*), 6.90 (d, J = 8.8 Hz, 2H, H-4'''), 6.98 (d, J = 4.9 Hz, 1H, H-5'), 7.35-7.38 (m, 4H, H-3, H-6 and H-3'''), 7.61 (d, J = 6.3 Hz, 1H, H-7), 8.30-8.32 (m, 2H, H-2 and H-6'), 8.56 (br d, J = 6.3 Hz, 1H, H-4), 10.16 (t, J = 5.1 Hz, 1H, 9-N*H*).

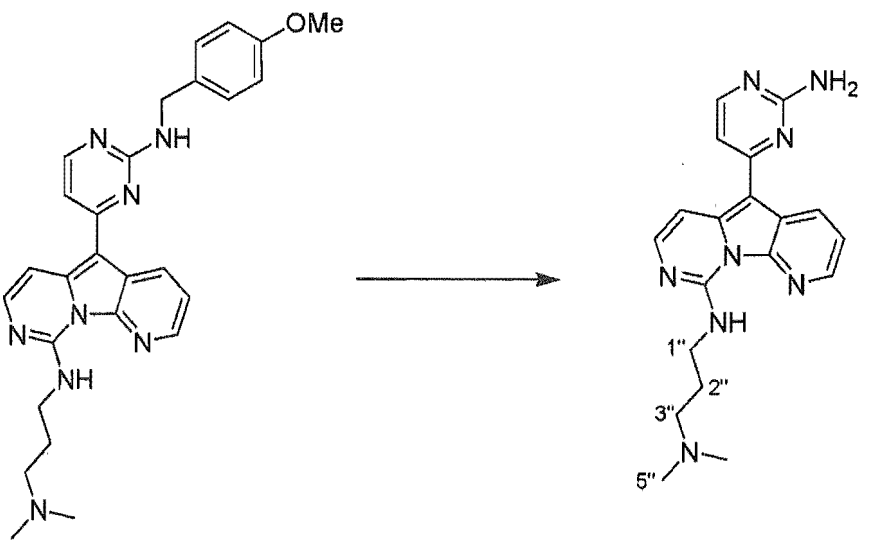
^{13}C NMR (75 MHz, $CDCl_3$): δ 27.0 (C-2''), 39.2 (C-1''), 45.1 (C-1'''), 45.3 (C-5''), 55.3 (OCH_3), 57.1(C-3''), 100.5 (C-5), 101.1 (C-6), 107.9 (C-5'), 113.9 (C-4'''), 120.0 (C-3), 121.9 (C-4a), 128.3 (C-4), 128.9 (C-3''') 131.5 (C-2'''), 138.5 (C-5a), 139.4 (C-2), 143.1 (C-7), 143.4 (C-10a), 149.1 (C-9), 157.4 (C-6'), 158.7 (C-5'''), 162.10 (C-2' or C-4'), 162.19 (C-2' or C-4').

IR (KBr): υ_{max} 2961, 2916, 2851, 1562, 1514, 1504, 1410, 1263, 1049, 1020, 802.

HRMS (ESI): Calcd for $C_{27}H_{31}N_8O$ (MH^+) *m/z* = 483.2621, found *m/z* = 483.2625.

IC_{50} (P388): 8.3 μM

9-[3-(Dimethylamino)propylamino]-5-[2-(amino)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.14



PMB-protected variolin **3.17** (13.0 mg, 27 μmol) was dissolved in triflic acid (300 μL) and the deep orange/red solution stirred at rt for 1 h. The solution was cooled to 0 °C and aq NH_3 added

dropwise until a yellow precipitate formed and the solution was alkaline. This suspension was loaded onto a C_{18} cartridge and washed with 60-80% MeOH/H_2O (0.1% TFA). The orange material thus obtained was dissolved in MeOH (0.5 mL), diluted with CH_2Cl_2 (10 mL), stirred with solid K_2CO_3 (during which time the solution lightened from an orange to a yellow colour), filtered and concentrated *in vacuo* to give the title compound as a thick yellow oil (5.9 mg, 60%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.99 (p, J = 6.8 Hz, 2H, H-2''), 2.31 (s, 6H, H-5''), 2.51 (t, J = 7.3 Hz, 2H, H-3''), 3.80 (q, J = 5.4 Hz, 2H, H-1''), 5.09 (br s, 2H, 2'-N*H*$_2$), 7.02 (d, J = 5.4 Hz, 1H, H-5'), 7.41-7.45 (m, 2H, H-3 and H-6), 7.67 (d, J = 6.3 Hz, 1H, H-7), 8.30 (d, J = 5.4 Hz, 1H, H-6'), 8.33 (dd, J = 1.5, 4.9 Hz, 1H, H-2), 8.68 (dd, J = 1.5, 8.3 Hz, 1H, H-4), 10.16 (t, J = 5.4 Hz, 1H, 9-N*H*).

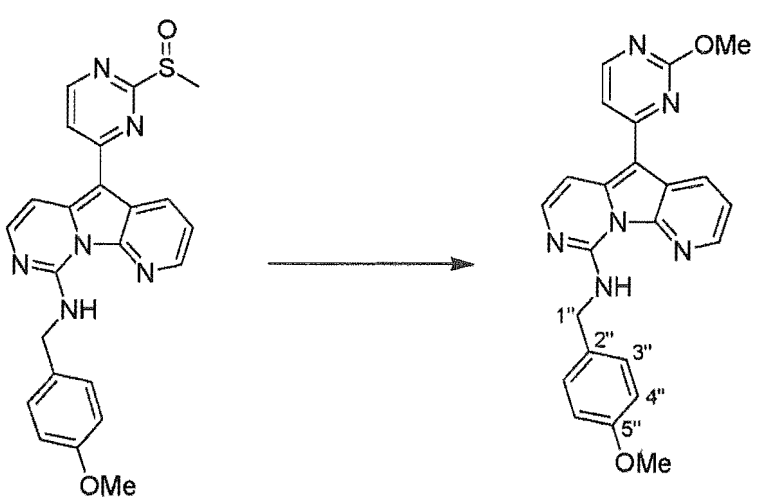
^{13}C NMR (75 MHz, $CDCl_3$): δ 27.1 (C-2''), 39.3 (C-1''), 45.5 (C-5''), 57.2(C-3''), 100.1 (C-5), 100.9 (C-6), 108.8 (C-5'), 120.1 (C-3), 121.9 (C-4a), 128.3 (C-4), 138.6 (C-5a), 139.5 (C-2), 143.4 (C-7), 143.5 (C-10a), 149.2 (C-9), 157.6 (C-6'), 162.6 (C- C-4'), 162.9 (C-2').

IR (KBr): υ_{max} 2943-2766 (series of weak bands), 1558, 1506, 1456, 1261, 1190, 1007, 810, 766.

HRMS (ESI): Calcd for $C_{19}H_{23}N_8$ (MH^+) *m/z* = 363.2046, found *m/z* = 363.2043.

IC_{50} (P388): 17 μM.

9-(4-Methoxybenzylamino)-5-[2-(methoxy)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.21



Monosulfoxide variolin **3.18** (6.6 mg, 15 μmol, assuming quantitative yields in prior oxidation and substitution reactions) was heated in a NaOMe/MeOH solution (1.37 M, 0.5 mL, 0.69 mmol of methoxide) at 105 °C for 20 h. The reaction mixture was sonicated periodically during the first 2 h to create a fine suspension. The mixture was allowed to cool to rt then concentrated *in vacuo*. The crude material thus obtained was purified by flash chromatography on silica, eluting with 40% EtOAc/hexanes, to give the title compound as a yellow amorphous solid (3.8 mg, 61% yield over 3 steps, ie, from core structure **2.6**).

Mp: 145-150 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.82 (s, 3H, PMB OC*H*$_3$), 4.14 (s, 3H, 2'-OC*H*$_3$), 4.91 (d, J = 5.9 Hz, 2H, 9-NHC*H*$_2$Ar), 6.92 (d, J = 8.8 Hz, 2H, PMB H-3''), 7.32 (d, J = 5.4 Hz, 1H, H-5'), 7.42 (d, J = 8.8 Hz, 2H, PMB H-2''), 7.45 (dd, J = 4.9, 8.3 Hz, 1H, H-3), 7.57 (d, J = 6.8 Hz, 1H, H-6), 7.76 (d, J = 6.3 Hz, 1H, H-7), 8.32 (dd, J = 1.5, 4.4 Hz, 1H, H-2), 8.49 (d, J = 5.4 Hz, 1H, H-6'), 8.75 (dd, J = 1.5, 8.3 Hz, 1H, H-4), 10.43 (t, J = 5.4 Hz, 1H, 9-N*H*).

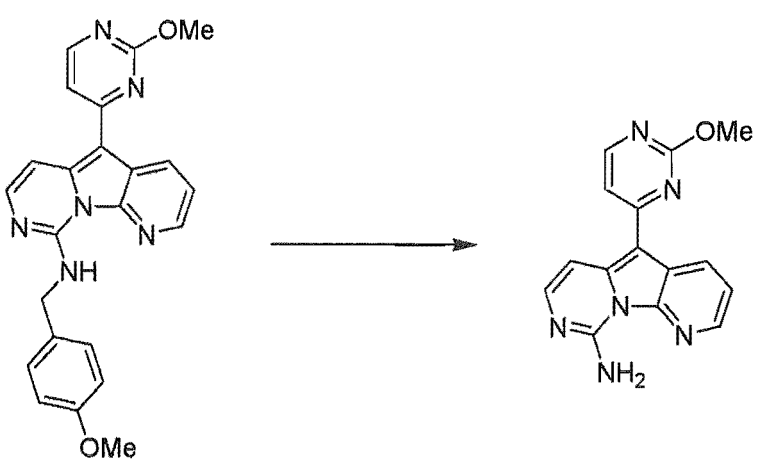
^{13}C NMR (75 MHz, $CDCl_3$): δ 44.4 (9-NHCH$_2$Ar), 54.7 (2'-OCH$_3$), 55.3 (PMB OCH$_3$), 100.0 (C-5), 101.5 (C-6), 111.2 (C-5'), 114.1 (PMB C-3''), 120.4 (C-3), 122.0 (C-4a), 128.5 (C-4), 128.9 (PMB C-2''), 130.2 (PMB C-1''), 139.3 (C-5a), 139.9 (C-2), 143.7 (C-10a), 144.1 (C-7), 149.3 (C-9), 158.6 (C-6'), 159.0 (PMB C-4''), 163.6 (C-4'), 165.6 (C-2').

IR (KBr): υ_{max} 3240-2710 (series of weak bands), 1701, 1581, 1358, 1249, 1134, 818, 521.

HRMS (ESI): Calcd for $C_{23}H_{21}N_6O_2$ (MH^+) *m/z* = 417.1726, found *m/z* = 413.1721.

IC$_{50}$ (P388): >30 μM

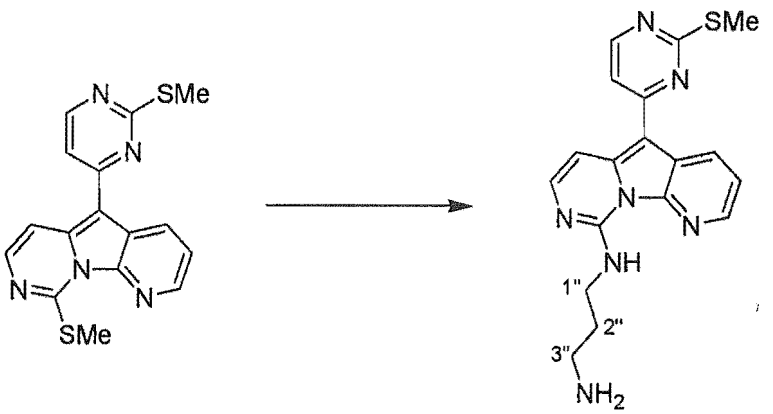
9-Amino-5-[2-(methoxy)pyrimidin-4-yl]pyrido[3',2';4,5]pyrrolo[1,2-*c*]pyrimidine 3.20



PMB-protected variolin **3.21** (3.8 mg, 9.2 μmol) was dissolved in triflic acid (100 μL) and the deep red/orange solution stirred at rt for 1 h. The solution was cooled to 0 °C and aq NH_3 added dropwise until a yellow precipitate formed and the solution was alkaline. This suspension was loaded onto a C_{18} cartridge and washed first with 1:1 MeOH/H_2O, then with MeOH to give the product as a thick yellow oil (0.9 mg, 90% pure, 30% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.14 (s, 3H, 2'-OC*H*$_3$), 7.34 (d, J = 5.4 Hz, 1H, H-5'), 7.52 (dd, J = 4.6, 8.1 Hz, H-3), 7.63 (m, 2H, H-6 and H-7), 8.43 (dd, J = 1.4, 4.9 Hz, 1H, H-2), 8.54 (d, J = 4.9 Hz, 1H, H-6'), 8.77 (dd, J = 1.5, 8.3 Hz, 1H, H-4).
HRMS (ESI): Calcd for $C_{15}H_{13}N_6O$ (MH^+) *m/z* = 293.1151, found *m/z* = 293.1148.
IC_{50} (P388): 3.6 μM

9-[3-(Amino)propylamino]-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.23



Deoxyvariolin core structure **2.6** (300 mg, 0.88 mmol) was heated with a 7.5 mol% 1,3-diaminopropane/toluene solution (4.91 mL, 4.42 mmol of the amine) at 100 °C for 16 h. (Water was rigorously excluded from the reaction, and the amine/toluene solution had been previously dried using a Dean-Stark apparatus.) The reaction solution was allowed to cool to rt then diluted with CH_2Cl_2 (50 mL), washed with 10% sat aq $NaHCO_3$ /sat brine solutions (50 mL), sat brine solution (50 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo* to give the title compound as thick orange gum (332 mg, 95% pure, 94% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.97 (p, J = 6.8 Hz, 2H, H-2''), 2.68 (s, 3H, SC*H*$_3$), 2.92 (t, J = 7.1 Hz, 2H, H-3''), 3.85 (q, J = 6.7 Hz, 2H, H-1''), 7.31 (d, J = 5.2 Hz, 1H, H-5'), 7.46 (dd, J = 4.8, 7.9 Hz, 1H, H-3), 7.48 (d, J = 6.7 Hz, 1H, H-6), 7.72 (d, J = 6.3 Hz, 1H, H-7), 8.35 (dd, J = 1.6, 4.8 Hz, 1H, H-2), 8.46 (d, J = 5.2 Hz, 1H, H-6'), 8.71 (dd, J = 1.2, 7.9 Hz, 1H, H-4), 10.15 (t, J = 5.2 Hz, 1H, 9-N*H*).

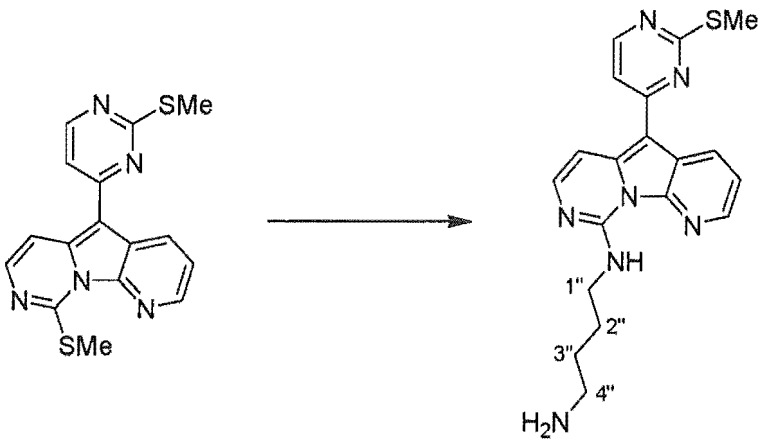
^{13}C NMR (75 MHz, $CDCl_3$): δ 14.3 (2'-S*C*H$_3$), 32.6 (C-2''), 38.3 (C-1''), 39.2 (C-3''), 99.8 (C-5), 101.2 (C-6), 112.5 (C-5'), 120.4 (C-3), 121.8 (C-4a), 128.5 (C-4), 138.9 (C-5a), 139.8 (C-2), 143.49 (C-10a), 143.58 (C-7), 149.5 (C-9), 156.3 (C-6'), 161.3 (C-4'), 172.2 (C-2').

IR ($CDCl_3$): υ_{max} 1686, 1558, 1489, 1258, 1184, 1134, 802, 725.

HRMS (ESI): Calcd for $C_{18}H_{20}N_7{}^{32}S$ (MH^+) *m/z* = 366.1501, found *m/z* = 366.1494.

IC_{50} (P388): 5.7 μM

9-[3-(Amino)butylamino]-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.24



Deoxyvariolin core structure **2.6** (30 mg, 88 μmol) was heated with a 32% 1,4-diaminobutane/toluene solution (420 μL, 1.32 mmol of the amine) at 90 °C for 20 h. (Water was rigorously excluded from the reaction, and the amine/toluene solution had been previously dried using a Dean-Stark apparatus.) The reaction solution was allowed to cool to rt, concentrated *in vacuo*, and the amine impurities removed by Kugelrohr distillation (120 °C, 0.03 mm Hg) to give the title compound as an orange gum (34 mg, 98% pure, 99% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.67 (p, J = 7.4 Hz, 2H, H-3''), 1.88 (p, J = 7.3 Hz, 2H, H-2''), 2.68 (s, 3H, SCH_3), 2.82 (br, 2H,H-4''), 3.75 (q, J = 6.7 Hz, 2H, H-1''), 7.29 (d, J = 5.3 Hz, 1H, H-5'), 7.46 (dd, J = 4.8, 8.2 Hz, 1H, H-3), 7.46 (d, J = 6.5 Hz, 1H, H-6), 7.71 (d, J = 6.4 Hz, 1H, H-7), 8.34 (dd, J = 1.4, 4.8 Hz, 1H, H-2), 8.45 (d, J = 5.6 Hz, 1H, H-6'), 8.69 (dd, J = 1.7, 8.2 Hz, 1H, H-4), 10.12 (t, J = 5.6 Hz, 1H, 9-N*H*).

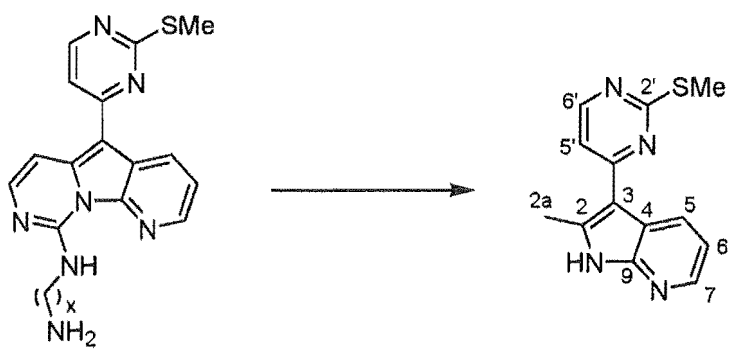
^{13}C NMR (75 MHz, $CDCl_3$): δ 14.3 (2'-SCH_3), 26.7 (C-2''), 31.2 (C-3''), 41.1 (C-1''), 41.8 (C-4''), 99.7 (C-5), 100.9 (C-6), 112.5 (C-5'), 120.4 (C-3), 121.9 (C-4a), 128.4 (C-4), 139.3 (C-5a), 139.7 (C-2), 143.6 (C-10a), 144.1 (C-7), 149.3 (C-9), 156.3 (C-6'), 161.5 (C-4'), 172.3 (C-2').

IR (KBr): υ_{max} 1682, 1558, 1489, 1256, 1184, 1138, 806, 772, 721.

HRMS (ESI): Calcd for $C_{19}H_{22}N_7{}^{32}S$ (MH^+) *m/z* = 380.1657, found *m/z* = 380.1660.

IC_{50} (P388): 21 μM

2-Methyl-3-(2-methylsulfanyl-pyrimidin-4-yl)-1H-pyrrolo[2,3-b]pyridine 3.25



If water was allowed to contaminate either of the above two reactions (synthesis of variolin analogues **3.24** and **3.25**), the degradation product shown above was isolated from the reaction mixture.

Mp: 175-185 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.66 (s, 3H, SCH_3), 2.90 (s, 3H,H-2a), 7.20-7.23 (m, 2H, H-5'and H-6), 8.32 (dd, J = 1.5, 5.0 Hz, 1H, H-7), 8.47-8.50 (m, 2H, H-6'and H-5), 11.59 (br s, 1H, N*H*).

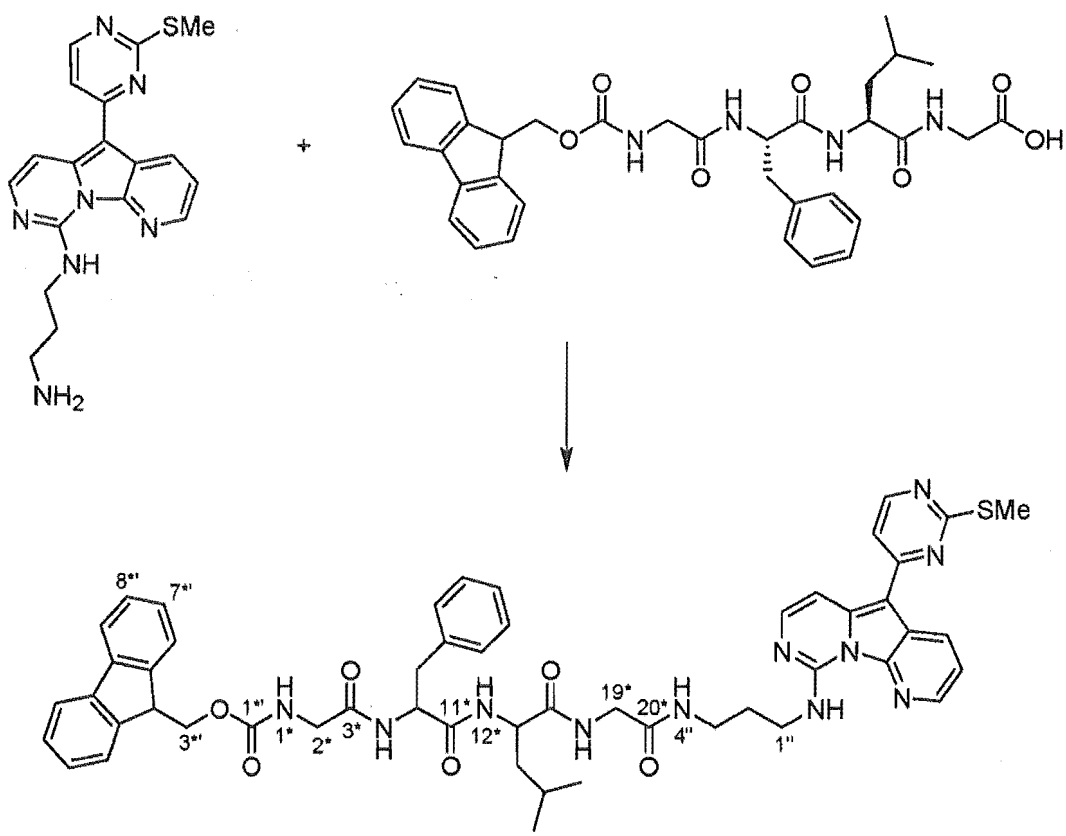
^{13}C NMR (75 MHz, $CDCl_3$): δ 14.2 (SCH_3), 15.0(C-2a), 110.9 (C-3), 113.5 (C-5'), 116.5 (C-6), 124.0 (C-4), 132.1 (C-7), 136.6 (C-5), 142.0 (C-9), 142.8 (C-2), 157.3 (C-6'), 160.2 (C-4'), 173.1 (C-2').

IR (KBr): υ_{max} 1686, 1558, 1339, 1281, 1138, 802, 672.

HRMS (EI): Calcd for $C_{13}H_{12}N_4S$ (M^+) *m/z* = 256.0783, found *m/z* = 256.0785.

IC_{50} (P388): 35 μM.

9-[3-{*N*-(9-Fluorenylmethoxycarbonyl)glycylphenylalanylleucylglycylamido}propylamino]-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.27



To a stirred solution of variolin analogue **3.23** (332 mg, 0.91 mmol), FmocGFLG (614 mg, 1.00 mmol), EDCI (226 mg, 1.18 mmol) and HOBT (184 mg, 1.36 mmol) in THF (60 mL) was added TEA (164 μL, 1.18 mmol), and the resulting yellow solution with colourless precipitate stirred at rt for 24 h. The suspension was washed with sat brine solution (2 × 100 mL), dried with Na_2SO_4, filtered and concentrated *in vacuo*. The crude material thus obtained was purified by flash chromatography on diol, eluting with 2% MeOH/CH_2Cl_2, to give the Fmoc-protected conjugate as an orange foam. Yield and melting point data were not able to be taken for this

conjugate due to the relatively large amount of solvent still present in the sample.* However, the yield was estimated to be ~65% based on ^{1}H NMR data.

* In an attempt to remove solvent residues from the sample, the conjugate material was heated to 70 °C under reduced pressure (0.03 mm Hg). This, however, led to the formation of a compound possessing very low solubility in all solvents investigated, thus disallowing the possibility for NMR characterisation.

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.82 (d, J = 6.3 Hz, 3H, H-16*a), 0.86 (d, J = 6.6 Hz, 3H, H-16*b), 1.50 (t, J = 7.2 Hz, 2H, H-14*), 1.58 (p, J = 6.3 Hz, 1H, H-15*), 1.87 (p, J = 7.0 Hz, 2H, H-2''), 2.59 (s, 3H, SC*H*$_3$), 2.77 (dd, J = 9.5, 13.7 Hz, 1H, H-6*a), 3.02 (dd, J = 4.2, 13.7 Hz, 1H, H-6*b), 3.25 (q, J = 6.3 Hz, 2H, H-3''), 3.51 (dd, J = 6.3, 17.0 Hz, 1H, H-2*a), 3.60-3.69 (m, 4H, H-1'', H-2*b and H-19a), 3.73 (dd, J = 6.0, 16.7 Hz, 1H, H-19*b), 4.15-4.28 (m, 4H, H-3*', H-4*' and H-13*), 4.54 (td, J = 4.2, 8.9 Hz, 1H, H-5*), 7.10 (m, 1H, H-10), 7.13-7.18 (m, 4H, H-8* and H-9*), 7.29 (t, J = 7.5 Hz, 2H, H-7*'), 7.38 (t, J = 7.4 Hz, 2H, H-8*'), 7.48 (d, J = 5.7 Hz, 1H, H-5'), 7.50-7.54 (m, 3H, H-1, H-3 and H-6), 7.66 (d, J = 7.5 Hz, 2H, H-6*'), 7.70 (d, J = 6.6 Hz, 1H, H-7), 7.84 (d, J = 7.5 Hz, 2H, H-9*'), 7.87 (t, J = 5.7 Hz, H-4''), 8.03 (d, J = 8.0 Hz, 1H, H-4*), 8.07 (t, J = 5.7 Hz, 1H, H-18*), 8.18 (d, J = 7.5 Hz, 1H, H-12*), 8.38 (dd, J = 1.2, 4.8 Hz, 1H, H-2), 8.48 (d, J = 5.7 Hz, 1H, H-6'), 8.71 (dd, J = 1.5, 8.3 Hz, 1H, H-4), 10.00 (t, J = 5.7 Hz, 1H, 9-N*H*).

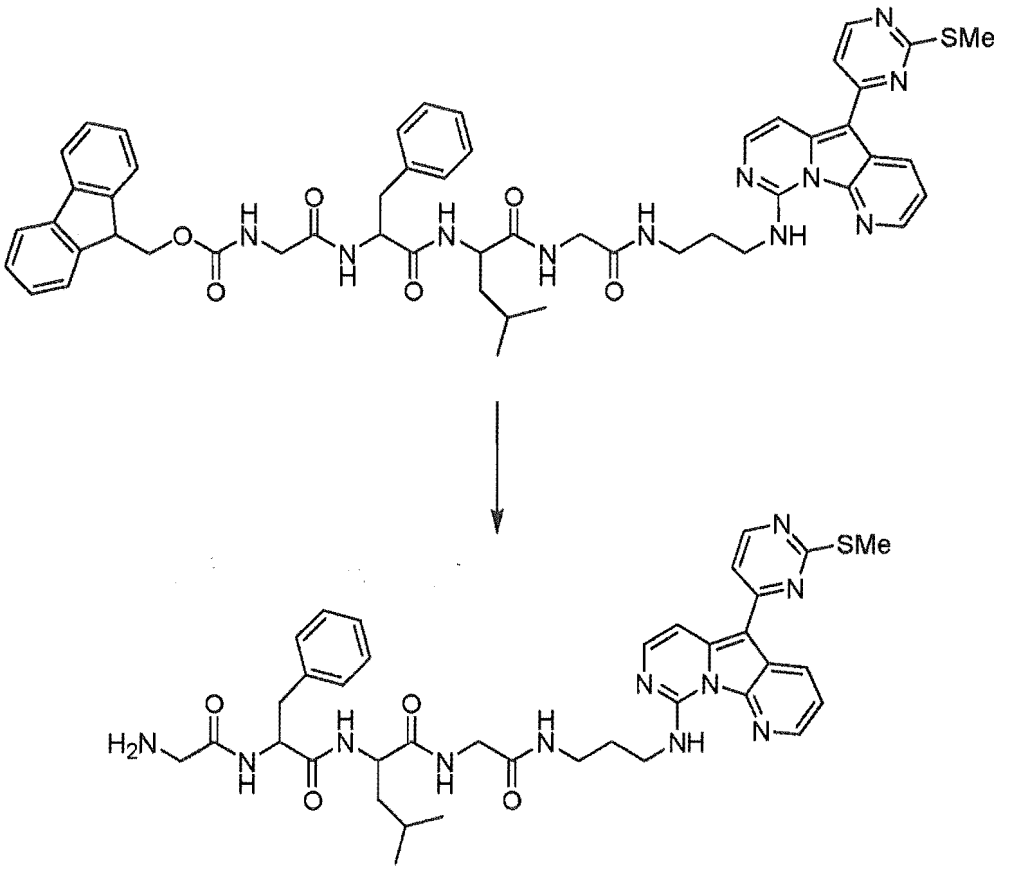
^{13}C NMR (75 MHz, $CDCl_3$): δ 13.8 (SC*H*$_3$), 21.8 (C-16*a), 23.1 (C-16*b), 24.2 (C-15*), 29.1 (C-2''), 36.4 (C-3''), 37.5 (C-6*), 38.5 (C-1''), 40.9 (C-14*), 42.3 (C-19*), 43.4 (C-2*), 46.7 (C-4*'), 51.6 (C-13*), 53.9 (C-5*), 98.6 (C-5), 101.0 (C-6), 112.7 (C-5'), 120.2 (C-9*'), 120.82 (C-3), 121.05 (C-4a), 125.3 (C-6*'), 126.3 (C-10'), 127.2 (C-7*'), 127.7 (C-8*'), 128.0 (C-9*), 128.6 (C-4), 129.3 (C-8*), 137.7 (C-7*), 139.1 (C-5a), 140.1 (C-2), 140.8 (C-10*'), 143.0 (C-10a), 143.9 (C-5*'), 144.5 (C-7), 148.7 (C-9), 156.6 (C-1*'), 156.9 (C-6'), 160.8 (C-4'), 168.8 (C-20*), 169.2 (C-3*), 171.08 (C-2'), 171.32 (C-11*), 172.2 (C-17).

IR (KBr): υ_{max}.3277, 3067, 2953, 1630, 1558, 1474, 1456, 1406, 1364, 1267, 1184, 1045, 1007, 978, 808, 743, 698.

HRMS (ESI): Calcd for (MH^+) *m/z* = 962.4136, found *m/z* = 962.3864.

IC_{50} (P388): 9.0 μM.

9-[3-(Glycylphenylalanylleucylglycylamido)propylamino]-5-[2-(methylsulfanyl)pyrimidin-4-yl]pyrido[3',2':4,5]pyrrolo[1,2-*c*]pyrimidine 3.26



The mixture of polymer **3.28** and deprotected conjugate **3.27** obtained in the previous reaction (530 mg, 0.58 mmol of conjugate) was dissolved in 2.5% piperidine/THF (40 mL) and stirred at rt for 40 min. Analysis of the reaction mixture by tlc showed the deprotection reaction to be complete. Hexanes (500 mL) were added with vigorous stirring to give a yellow gel-like precipitate in a very light yellow solution. This suspension was left at -20 °C for 3 h, then the precipitate collected by filtration. This material was dissolved in THF (50 mL) to give a light orange solution with a fine colourless precipitate, which was filtered, concentrated *in vacuo* and heated to 70 °C under reduced pressure (0.03 mm Hg) for 48 h to afford the deprotected conjugate as an orange foam (412 mg, 95% pure, 62% yield over 2 steps, ie, from variolin analogue **3.23**).

Mp: 109-111 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.83 (d, J = 6.4 Hz, 3H, H-16*a), 0.87 (d, J = 6.4 Hz, 3H, H-16*b), 1.49 (t, J = 7.3 Hz, 2H, H-14*), 1.57 (p, J = 6.7 Hz, 1H, H-15*), 1.87 (p, J = 7.8 Hz,

H-2''), 2.61 (s, 3H, SC*H*$_3$), 2.80 (dd, J = 8.8, 13.7 Hz, 1H, H-6*a), 3.01 (m, 3H, H-6*b and H-2*), 3.24 (m, 2H, H-3''), 3.60-3.76 (m, 4H, H-1'' and H-19*), 4.25 (q, J = 7.7 Hz, 1H, H-13*), 4.57 (m, 1H, H-5*), 7.09-7.18 (m, 5H, H-8*, H-9* and H-10*), 7.57-7.62 (m, 3H, H-3, H-5' and H-6), 7.76 (d, J = 6.4 Hz, 1H, H-7), 7.90 (t, J = 5.4 Hz, 1H, H-4''), 8.01 (br, 1H, H-4*), 8.13 (t, J = 5.9 Hz, 1H, H-18*), 8.25 (d, J = 7.3 Hz, 1H, H-12*), 8.45 (dd, J = 1.5, 4.9 Hz, 1H, H-2), 8.52 (d, J = 5.4 Hz, 1H, H-6'), 8.80 (dd, J = 1.0, 7.8 Hz, 1H, H-4), 10.06 (t, J = 5.9 Hz, 1H, 9-N*H*).

^{13}C NMR (75 MHz, $CDCl_3$): δ 14.0 (SC*H*$_3$), 21.8 (C-16*a), 23.2 (C-16*b), 24.3 (C-15*), 29.1 (C-2''), 36.4 (C-3''), 37.8 (C-6*), 38.6 (C-1''), 40.7 (C-14*), 42.3 (C-19*), 44.5 (C-2*), 51.7 (C-13*), 53.5 (C-5*), 101.1 (C-6), 112.8 (C-5'), 121.01 (C-3 or C-4a), 121.15 (C-3 or C-4a), 126.4 (C-10*), 128.2 (C-9*), 128.6 (C-4), 129.4 (C-8*), 137.5 (C-7*), 139.2 (C-5a), 140.3 (C-2), 144.7 (C-7), 148.9 (C-9), 157.1 (C-6'), 160.9 (C-4'), 169.0 (C-20*), 171.2 (C-2'), 171.5 (C-11*), 172.4 (C-17*), 172.9 (C-3*).

IR (KBr): υ_{max}.3281, 3067, 2957, 2870, 1653, 1558, 1472, 1406, 1364, 1265, 1207, 1184, 1057, 1007, 976, 910, 808, 768, 700.

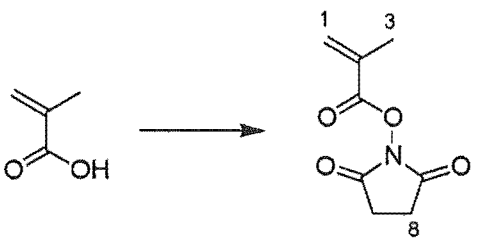
$\varepsilon_{(410\ nm)}$: 20000 $Lmol^{-1}cm^{-1}$

HRMS (ESI): Calcd for (MH^+) *m/z* = 740.3455, found *m/z* = 740.3476.

IC_{50} (P388): 9.9 μM

6.4 Experiments described in Chapter 4

Methacryloxysuccinimide (MAOS)



This was synthesised via the slightly modified protocols of Chang.[145] To a solution of methacrylic acid (1.00 mL, 11.8 mmol) and NHS (1.36 g, 11.8 mmol) in THF (15 mL) at 0 °C was added DCC (2.676 g, 13.0 mmol), and the solution/suspension stirred at 0 °C for 1 h. The white suspension was then allowed to warm to room temperature and stirred for a further 1 h, before being filtered, the residues washed with EtOAc (30 mL), and the pooled filtrates concentrated *in vacuo*. The resulting crude material was dissolved in boiling EtOAc (50 mL) and filtered while hot. The filtrate was concentrated *in vacuo*, and the residues dissolved in boiling EtOAc (30 mL), filtered while hot, and concentrated *in vacuo*. The resulting residues were again dissolved in boiling EtOAc (10 mL), filtered while hot, and concentrated *in vacuo*. The resulting material was recrystallised from EtOAc/hexanes to afford MAOS in high purity as fine white crystals (1.1295 g, 52% yield).

Mp: 73-75 °C.

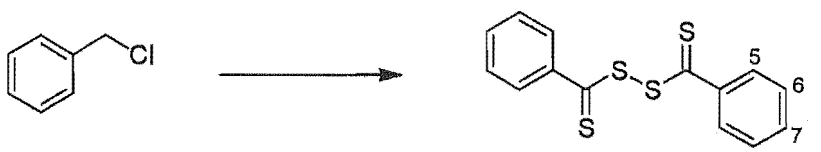
^{1}H NMR (500 MHz, $CDCl_3$): δ 2.06 (t, J = 1.5 Hz, 3H, H-3), 2.86 (s, 4H, H-8), 5.89 (d, J = 1.0 Hz, 1H, H-1a), 6.42 (t, J = 1.3 Hz, 1H, H-1b).

Copper(I) chloride[146]

To a solution of $CuSO_4.5H_2O$ (3.480 g) and NaBr (1.506 g) in H_2O (20 mL) was added, over 2 min, a solution of Na_2SO_3 (1.791 g) in H_2O (15 mL). The resulting pale blue/green solution with white precipitate was poured into a solution of conc. HCl (0.7 mL) and Na_2SO_3 (0.35 g) in H_2O (350 mL). The suspension was stirred thoroughly, filtered, the precipitate washed with

glacial acetic acid (5 × 10 mL), EtOH (3 × 10 mL) and diethyl ether (3 × 10 mL), then dried *in vacuo* to give the title compound as an off-white solid (1.190 g, 60% yield).

Bis(thiobenzyl)disulfide[147]

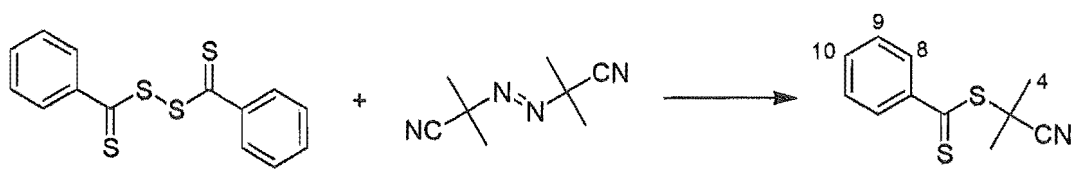


To a yellow suspension of elemental sulfur (1.01 g, 31.5 mmol) in sodium methoxide/methanol (2.0 M, 15 mL, 30.0 mmol of NaOMe) was added benzyl chloride (1.82 mL, 15.8 mmol) over 1 h with stirring. The resulting brown solution/suspension was heated to reflux for 20 h, allowed to cool to rt, filtered, and the filtrate concentrated *in vacuo*. The red oil thus obtained was dissolved in H_2O (16 mL), washed with diethyl ether (3 × 8 mL), acidified with 32% aq HCl (5 mL) and extracted with diethyl ether (8 mL). The deep purple organic solution was extracted with 0.67 M aq NaOH (3 × 55 mL). The resulting purple-coloured aqueous solutions of sodium dithiobenzoate were combined, and 0.2 M aq potassium ferricyanide (250 mL, 50 mmol) added by dripping funnel over 45 min with vigorous stirring at rt. The resulting red precipitate was collected by filtration, washed with H_2O, and dried under reduced pressure (0.03 mm Hg). Recrystallisation from EtOAc gave bis(thiobenzyl)disulfide as red crystals (740 mg, 31% yield).

Mp: 90-91 °C (lit. 91-2 °C).[148]

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.46 (tt, J = 1.8, 7.9 Hz, 4H, H-6), 7.61 (tt, J = 1.2, 7.5 Hz, 2H, H-7), 8.09 (dd, J = 1.2, 8.3 Hz, 4H, H-5).

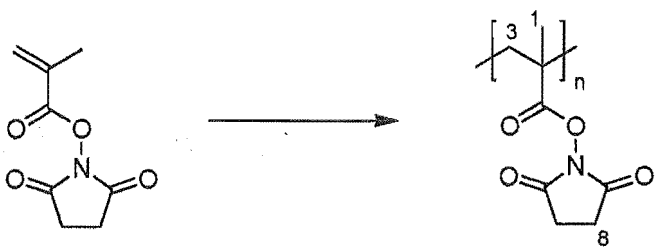
2-(2-Cyanopropyl)dithiobenzoate (CPDB) 4.1[147]



Bis(thiobenzyl)disulfide (740 mg, 2.41 mmol) and AIBN (595 mg, 3.62 mmol) were dissolved in EtOAc (15 mL) and heated to reflux for 24 h. The red solution was allowed to cool to rt, concentrated *in vacuo* and the crude material purified by flash chromatography on silica, eluting with 2% EtOAc/hexanes, to give the title compound as a red oil (699 mg, 87% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.93 (s, 6H, H-4), 7.39 (tt, J = 1.8, 7.9 Hz, 2H, H-9), 7.55 (tt, J = 1.3, 7.3 Hz, 1H, H-10), 7.91 (dd, J = 0.7, 8.7, 2H, H-8).

Poly-methyacryloxysuccinimide (pMAOS)



a) Atom-transfer radical polymerisation (ATRP):

This was synthesised via the slightly modified protocols of Godwin *et al.*[60] To MAOS (1.00 g, 5.46 mmol), CuBr (7.0 mg, 49 μmol) and 2,2'-bipyridine (15.3 mg, 98 μmol) in a Young's tube was added DMSO (700 μL) and the resulting solution degassed with three successive freeze-pump-thaw cycles and left under argon. Ethyl-2-bromoisobutyrate (7.2 μL, 49 μmol) was added under a flow of argon, and the solution left in an oven at at 90 °C for 4 h, during which time it changed from a pale green/blue to a darker green colour. DMF (2 mL) was added to the viscous solution, which was allowed to cool to rt before being poured into acetone (10 mL) with vigorous stirring. The white precipitate which formed immediately was collected by filtration, washed with acetone, and redissolved in DMF (10 mL). To the resulting solution was slowly added acetone (40 mL), and the resulting precipitate collected, washed with acetone, and left under reduced pressure (0.03 mm Hg, 3 d) to afford pMAOS as a white powder (661 mg, 66% yield).

^{1}H NMR (500 MHz, d_6-DMSO): δ 1.0-1.8 (br, 3H, H-1), 1.9-? (br, 2H, H-3), 2.6-3.1 (br, 4H, H-8).

IR (KBr): υ_{max} 2997, 2947, 1778, 1740, 1429, 1364, 1205, 1065, 814, 650.

M_{target}: 13.4 kDa

$\bar{M}_n$: 25 kDa

$\bar{M}_n/\bar{M}_w$: 1.87

b) Reversible addition-fragmentation chain transfer (RAFT) polymerisation:

This was synthesised via the slightly modified protocols of Schilli *et al.*[120] To MAOS (1.331 g, 7.27 mmol) and CPDB (91 mg, 414 μmol) in a Young's tube was added DMF (7.3 mL) and the resulting solution degassed with three successive freeze-pump-thaw cycles and left under argon. AIBN (6.8 mg, 41 μmol) was added under a flow of argon, and the solution stirred at 60 °C for 16 h. The red solution was allowed to cool to rt and the polymer precipitated with MeOH (25 mL). The precipitate was collected by filtration, redissolved in hot DMF (5 mL), and MeOH (25 mL) added. The resulting precipitate was collected by filtration and traces of solvent removed *in vacuo* to give pMAOS as a light pink powder (1.20 g, 90 % yield).

M_{target}: 2.6 kDa

$\bar{M}_n$: 30 kDa

$\bar{M}_n/\bar{M}_w$: 1.36

Measuring extinction coefficient of variolin conjugate 3.26

a) Using UV spectroscopy:

Three solutions of variolin conjugate **3.26** in DMSO (1.0 mg/mL) were generated. An aliquot (80 μL) of each solution was diluted into DMSO (1.92 mL); these dilutions were performed in triplicate to give a total of 9 solutions of conjugate **3.26** in DMSO (2 mL, 54 μM). The absorbancy of each of these solutions was measured at 410 nm, with the average of the 9 readings (which were all within 2% of the average) being $A_{(410)} = 1.08$ cm^{-1}. This figure gives $\varepsilon_{(410)} = 20 \times 10^3$ L.mol^{-1}cm^{-1}.

b) Using the HPLC:

Three solutions of variolin conjugate **3.26** in DMSO (0.25 mg/mL) were generated. Two aliquots (10 and 50 μL) of each solution were injected onto the HPLC and analysed using the

standard gradient elution protocol at 248 nm. The average of the six values (which were in close agreement) gave an 'extinction coefficient' at 248 nm of 1.03×10^{12} AU.min.mol^{-1}. Also, the 'extinction coefficient' at 410 nm was 1.47×10^{11} AU.min.mol^{-1}.

Attempted syntheses of polymer therapeutics incorporating analogue 3.23

a) From pMAOS:

A solution of variolin conjugate **3.26** (7.9 mg, 11 μmol) and pMAOS (20 mg, 109 μmol) in DMSO (500 μL) was stirred under argon at 60 °C for 2 h. 1A2P (17 μL, 215 μmol) was added and the yellow solution stirred for a further 3 h at 60 °C. The solution was allowed to cool to rt, diluted with MeOH (500 μL) and subjected to LH20 chromatography, eluting with MeOH. The fast-eluting, high Mr fraction was collected and concentrated *in vacuo* to give the polymer as a light yellow solid (11 mg, ~70% yield). The slower eluting, low Mr material was collected as six separate fractions, which were concentrated *in vacuo* to give amorphous solids, some of which were yellow in colour.

b) From ring-opened copolymer 4.3 (formed in situ from pMAOS) at high temperature:

A solution of pMAOS (27 mg, 149 μmol) and 1A2P (13 μL, 164 μmol) in dry DMSO (500 μL) was stirred under argon at 70 °C for 4 h. To this solution was added, via cannula, a solution of variolin conjugate **3.26** (11 mg, 15 μmol) in DMSO (600 μL), and the resulting yellow solution stirred at 70 °C for 22 h. 1A2P (24 μL, 297 μmol) was added and the yellow solution stirred at 70 °C for an additional 17 h. The solution was allowed to cool to rt, diluted with MeOH (1 mL), and subjected to LH20 chromatography, eluting with MeOH. The fast-eluting, high Mr fraction was collected and concentrated *in vacuo* to give the polymer as a light yellow solid (19 mg, ~85% yield). The slower eluting, low Mr material was collected and concentrated *in vacuo*. A solution of the polymeric material in DMSO (0.25 mg/mL) was generated to allow UV quantification of the variolin material on the polymer backbone. The solution was generated in triplicate, and the readings were taken in triplicate at 410 nm to give an average absorbancy of 1.024 cm^{-1}. The low Mr material was dissolved in DMSO (3 mL), an aliquot removed and diluted 1:100 into DMSO, and an aliquot (100 μL) of the resulting solution injected onto the HPLC and analysed using the standard gradient elution protocol.

c) From ring-opened copolymer 4.3 at low temperature:

A solution of ring-opened copolymer **4.3** (x = 0.55, y = 0.45, 47 mg, 270 μmol), variolin conjugate **3.26** (20 mg, 27 μmol) and TEA (19 μL, 137 μL) in DMSO (500 μL) was stirred under argon at 45 °C.* Aliquots (10 μL) were removed after 1, 2 and 7 h, diluted into DMSO (1 mL), and an aliquot (10 μL) of the resulting yellow solution injected onto the HPLC and analysed using the standard gradient elution protocol. From the solution that had been created by removal of an aliquot after 7 h was removed an aliquot (100 μL) which was further diluted into DMSO (0.9 mL). The UV absorbancy of this solution was measured in triplicate to give an average $A_{(410\ nm)} = 1.012\ cm^{-1}$. After 7 h at 45 °C, the resulting yellow solution was allowed to cool to rt, diluted with MeOH (1 mL) and subjected to LH20 chromatography, eluting with MeOH. The fast-eluting deep yellow high Mr fraction was collected and concentrated *in vacuo* to give the copolymer as a light yellow powder (46 mg, ~90% yield). Analysis of this material by ^{1}H NMR spectroscopy indicated a >5% molar loading of variolin analogue **3.23**, with 40 mol% hydroxamate material. A solution of the copolymer in DMSO (0.1 mg/mL) was generated, and the absorbancy measured in triplicate to give an average $A_{(410)} = 0.585\ cm^{-1}$. The faint yellow low Mr material was collected, concentrated *in vacuo* and shown by HPLC and LCMS to contain a small amount of conjugate **3.26** and other compounds bearing a variolin-like chromophore.

A solution of the newly-formed copolymer (40 mg, ~215 μmol), 1A2P (36 μL, 455 μL) and TEA (80 μL, 577 μL) in DMSO (500 μL) was stirred under argon at 45 °C for 19 h. The resulting yellow solution was allowed to cool to rt, diluted with MeOH (1 mL) and subjected to LH20 chromatography, eluting with MeOH. The fast-eluting yellow high Mr fraction was collected and concentrated *in vacuo* to give the polymer as a light yellow water soluble powder (yield not taken). Analysis by ^{1}H NMR spectroscopy confirmed the presence of variolin material, aswell as 5-10% polymer-bound hydroxamate material. A solution of the polymer in DMSO (0.1 mg/mL) was generated and the UV absorbancy measured to give $A_{(410)} = 0.700\ cm^{-1}$. HPLC analysis showed the sample to be free of low Mr material. The pale yellow low Mr material was

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* At t = 0 an aliquot (10 μL) was removed, diluted 1:1000 into DMSO, and the UV absorbance of the resulting solution measured to give $A_{(410\ nm)} = 1.091\ cm^{-1}$.

collected, concentrated *in vacuo*, and shown by HPLC to contain small amounts of three conjugate-like compounds.

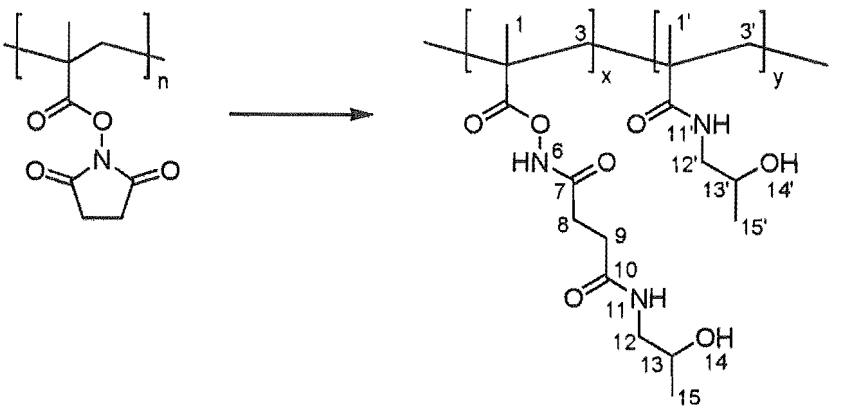
IC_{50} (P388) of the copolymer: >125 μg/mL.

d) From ring-opened copolymer 4.3 at low temperature:

A solution of ring-opened copolymer **4.3** (9.4 mg, 45 μmol), variolin conjugate **3.26** (5.0 mg, 6.8 μmol) and TEA (1.2 μL, 8.7 μmol) in DMSO (500 μL) was stirred under argon at 45 °C for 5 h. 1A2P (36 μL, 455 μmol) was added and the yellow solution stirred at 45 °C for 48 h. The solution was allowed to cool to rt, diluted with MeOH (0.5 mL) and subjected to LH20 chromatography, eluting with MeOH. The high Mr material was collected and concentrated *in vacuo* to give a non-water soluble powder (yield not taken). Analysis by ^{1}H NMR spectroscopy showed the polymer to be ~50% comprised of glutarimide material.

IC_{50} (P388) of the copolymer: >125 μg/mL.

Ring-opened copolymer 4.3

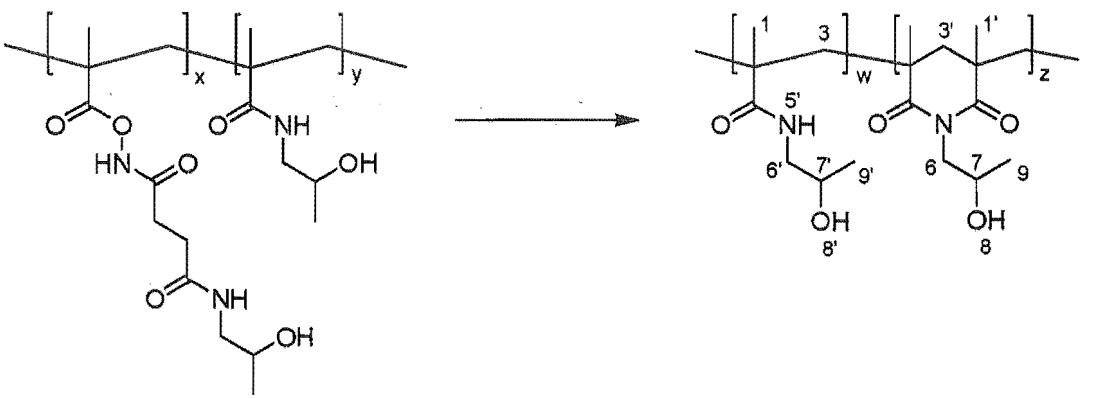


This reaction was performed many times, with the following conditions being a typical example. pMAOS (100 mg, 546 μmol) was stirred with 1A2P (52 μL, 658 μmol) in dry DMSO (500 μL) for 3.5 h at 45°C. Chromatography on LH20, eluting with MeOH, afforded the copolymer as a clear colourless glass (x = 0.6, y = 0.4, 116 mg, 82% yield).

^{1}H NMR (500 MHz, d_6-DMSO): (all peaks are broad) δ 0.7-1.4 (H-1 and 1'), 1.1-1. 2 (H-15 and H-15'), 1.4-2.4 (H-3 and H-3'), 2.3-2.5 (H-8 and H-9), 2.7-3.1 (H-12'), 2.9-3.1 (H-12), 3.6-3.7 (H-13), 3.6-3.8 (H-13'), 4.6-4.7 (H-14), 4.6-4.8 (H-14'), 7.1-7.5 (H-11'), 7.8-7.9 (H-11), 11.5-11.8 (H-6). Integrals: H-1, H-1', H-3, H-3', H-15 and H-15' = 7.67; H-8 and H-9 = 2.20; H=12 and H-12' = 2.10; H-13 and H-13' = 1.05; H-14 and H-14' = 0.86; H-11' = 0.34; H-11 = 0.52; H-6 = 0.54.

IR (KBr): υ_{max} (all signals are weak) 2930, 1767, 1695, 1539, 1373, 1261, 1080.

Glutarimide copolymer 4.6



a) In the absence of 1A2P:

A solution of ring-opened copolymer **4.3** (x = 0.35, y = 0.65; 35mg, 191 μmol) in d_6-DMSO (200 μL) was heated at 70 °C for 24 h, and examined periodically by ^{1}H NMR. Over 24 h the broad polymeric hydroxamate peaks of copolymer **4.3** were replaced by the sharper, more well-defined peaks of hydroxamic acids **4.4** and **4.5**. The reaction mixture was subjected to LH20 chromatography, eluting with MeOH, and the high-molecular weight fractions collected and concentrated *in vacuo* to afford copolymer **4.6** as a clear colourless glass (w = 0.45, z = 0.55; 19 mg, 85 % yield).

b) In the presence of 1A2P:

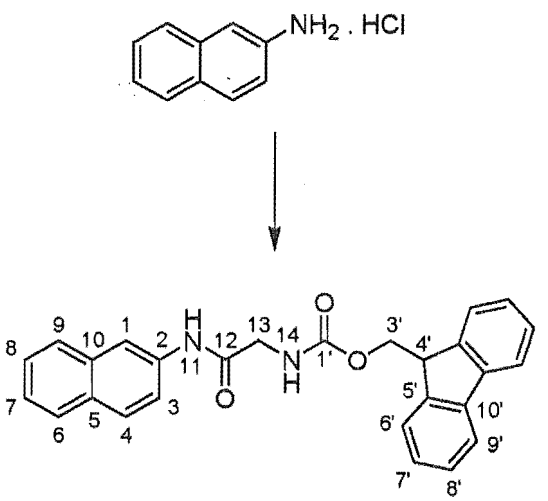
Ring-opened copolymer **4.3** (x = 0.55, y = 0.45; 10.0 mg, 48 μmol) was stirred with 1A2P (250 μL, 3.2 mmol) in dry DMSO (250 μL) for 24 h at 45°C. Chromatography on LH20, eluting

with MeOH, afforded copolymer **4.6** as a clear colourless glass (w = 0.55, z = 0.45; 5.2 mg, 88% yield).

^{1}H NMR (500 MHz, d_4-methanol): (all signals are broad) δ 0.9-1.5 (H-1, H-1', H-9 and H-9'), 1.5-2.5 (H-3 and H-3'), 2.9-3.3 (H-6'), 3.4-3.9 (H-6), 3.8-4.0 (H-7 and H-7'), 7.5-7.7 (H-5'). Integrals: H-1, H-1', H-9 and H-9' = 5.50; H-3 and H-3' = 2.00; H-6' = 0.60; H-6, H-7 and H-7' = 1.05; H-5' = 0.33.

IR (KBr): υ_{max} (all signals are weak) 2968, 2934, 1719, 1533, 1380, 1194, 1090, 101, 889.

_N_-(9-Fluorenylmethoxycarbonyl)-glycyl-β-naphthylamide



β-Naphthylamine hydrochloride salt (60 mg, 0.33 mmol), Fmoc-glycine (104 mg, 0.35 mmol), DCC (90 mg, 0.43 mmol), HOBT (68 mg, 0.50 mmol) and TEA (51 μL, 0.37 mmol) were dissolved in THF (5 mL) and the solution/suspension stirred at rt for 19 h. The resulting fine white suspension was concentrated *in vacuo* and the crude material thus obtained purified by flash chromatography on silica, eluting with 10% EtOAc/hexanes, to afford the title compound as a white solid (125 mg, 88% yield).

Mp: 176-177 °C.

^{1}H NMR (500 MHz, d_6-DMSO): δ 3.86 (d, J = 6.4 Hz, 2H, H-13), 4.26 (t, J = 6.8 Hz, 1H, H-4'), 4.32 (d, J = 7.3 Hz, 2H, H-3'), 7.35 (t, J = 7.6 Hz, 2H, H-7'), 7.38-7.48 (m, 4H, H-7, H-8 and H-8'), 7.58 (dd, J = 2.0, 8.8 Hz, 1H, H-3), 7.68 (t, J = 6.1 Hz, 1H, H-14), 7.74 (d, J = 7.3 Hz, 2H,

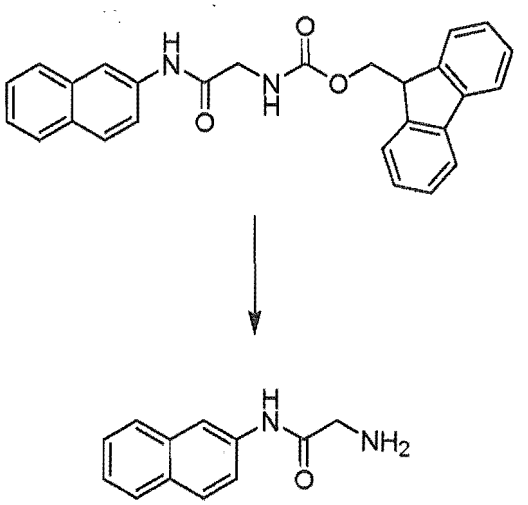
H-6'), 7.80 (d, J = 8.3 Hz, 1H, H-9), 7.83 (d, J = 8.3 Hz, 1H, H-6), 7.86 (d, J = 8.8 Hz, 1H, H-4), 7.91 (d, J = 7.3 Hz, 1H, H-9'), 8.27 (s, 1H, H-1), 10.18 (s, 1H, H-11).

^{13}C NMR (75 MHz, d_6-DMSO): δ 44.2 (C-13). 46.8 (C-4'), 65.9 (C-3'), 115.3 (C-1), 120.0 (C-3), 120.3 (C-9'), 124.8 (C-8), 125.4 (C-6'), 126.6 (C-7), 127.2 (C-7'), 127.4 (C-9), 127.6 (C-6), 127.8 (C-8'), 128.6 (C-4), 129.9 (C-10), 133.6 (C-5), 136.6 (C-2), 140.9 (C-10'), 144.0 (C-5'), 156.8 (C-1'), 168.4 (C-12).

IR (KBr): υ_{max} 3335, 3042, 1684, 1591, 1539, 1450, 1398, 1352, 1285, 1236, 1150, 1042, 856, 816, 739.

HRMS (ESI): Calcd for $C_{27}H_{23}N_2O_3$ (MH^+) *m/z* = 423.1709, found *m/z* = 423.1723.

Glycyl-β-naphthylamide



Fmoc-Glycyl-β-naphthylamide (305 mg, 0.72 mmol) was dissolved in 10% piperidine/THF (10 mL) and stirred at rt for 30 min. Hexanes (200 mL) were added with vigorous stirring, and the resulting white suspension left at -20 °C overnight. The precipitate was collected by filtration and washed with hexanes to give the title compound as a white solid (120 mg, 83% yield).

Mp: 118-120 °C.

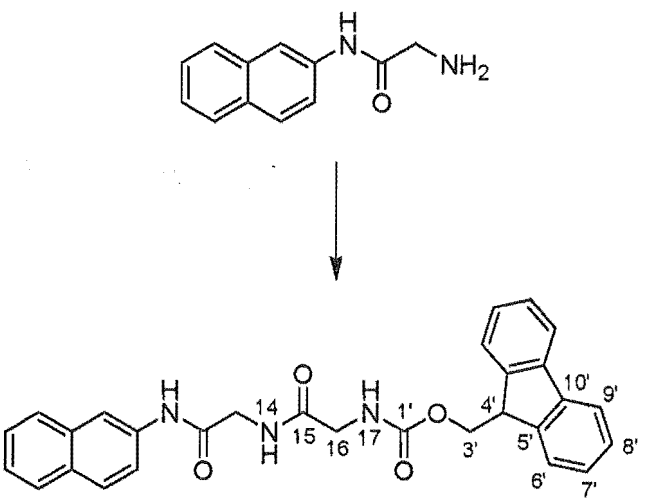
^{1}H NMR (500 MHz, d_6-DMSO): δ 3.33 (s, 2H, H-13), 7.39 (t, J = 7.6 Hz, 1H, H-8), 7.46 (t, J = 7.6 H, 1H, H-7), 7.65 (dd, J = 2.0, 8.8 Hz, 1H, H-3), 7.80-7.87 (m, 3H, H-4, H-6 and H-9), 8.33 (s, 1H, H-1), 9-11 (br s, 1H, H-11).

^{13}C NMR (75 MHz, d_6-DMSO): δ 45.7 (C-13), 115.0 (C-1), 120.0 (C-3), 124.6 (C-8), 126.5 (C-7), 127.4 (C-6), 127.6 (C-9), 128.5 (C-4), 129.8 (C-10), 133.6 (C-5), 136.6 (C-2), 170.0 (C-12).

IR (KBr): υ_{max} 3059, 1663, 1591, 1570, 1506, 1472, 1367, 1265, 1244, 966, 941, 891, 856, 810, 746.

HRMS (ESI): Calcd for $C_{12}H_{13}N_2O$ (MH^+) *m/z* = 201.1028, found *m/z* = 201.1030.

_N_-(9-Fluorenylmethoxycarbonyl)-glycylglycyl-β-naphthylamide



Glycyl-β-naphthylamide (112 mg, 0.56 mmol), Fmoc-glycine (166 mg, 0.56 mmol), DCC (150 mg, 0.73 mmol) and HOBT (113 mg, 0.84 mmol) were dissolved in THF (20 mL) and stirred at room temperature for 18 h. To the resulting white suspension was added THF (30 mL) and silica (5 g), and the resulting slurry sonicated for 5 min then stirred at rt for 8 h. Hexanes (50 mL) were added slowly with vigorous stirring, and the slurry then loaded onto a column of silica in CH_2Cl_2. Elution with 2:1 CH_2Cl_2/EtOAc (2% MeOH) followed by warm 2% MeOH/THF afforded the protected dipeptide conjugate as a white solid (326 mg, ~80% pure, quantitative yield).

Mp: 189-193 °C.

^{1}H NMR (500 MHz, d_6-DMSO): δ 3.71 (d, J = 5.9 Hz, 2H, H-16), 3.96 (d, J = 5.9 Hz, 1H, H-13), 4.25 (t, J = 7.1 Hz, 1H, H-4'), 4.32 (d, J = 7.3 Hz, 2H, H-3'), 7.33 (t, J = 7.8 Hz, 2H, H-7'), 7.40-7.43 (m, 3H, H-8 and H-8'), 7.46 (dt, J = 1.0, 7.3 Hz, 1H, H-7), 7.62 (dd, J = 2.0, 8.8 Hz,

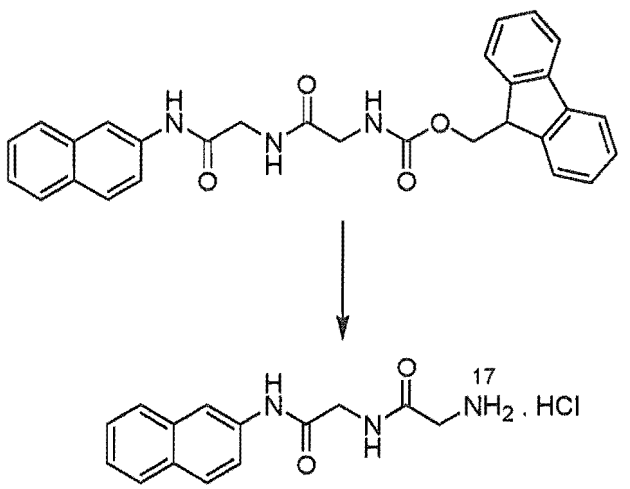
1H, H-3), 7.88 (t, J = 6.1 Hz, 1H, H-17), 7.72 (d, J = 7.3 Hz, 2H, H-6'), 7.78 (d, J = 7.8 Hz, 1H, H-9), 7.83 (d, J = 8.3 Hz, 1H, H-6), 7.86 (d, J = 8.8 Hz, 1H, H-4), 7.89 (d, J = 7.3 Hz, 2H, H-9'), 8.26 (s, 1H, H-1), 8.29 (t, J = 5.6 Hz, 1H H-14), 10.16 (s, 1H, H-11).

^{13}C NMR (75 MHz, d_6-DMSO): δ 42.9 (C-13), 43.7 (C-16), 46.8 (C-4'), 66.0 (C-3'), 115.5 (C-1), 120.1 (C-3), 120.3 (C-9'), 124.9 (C-8), 125.4 (C-6'), 126.7 (C-7), 127.3 (C-7'), 127.4 (C-9), 127.7 (C-6), 127.8 (C-8'), 128.6 (C-4), 130.0 (C-10), 133.5 (C-5), 136.5 (C-2), 140.9 (C-10'), 144.0 (C-5'), 156.9 (C-1'), 168.1 (C-12), 169.9 (C-15).

IR (KBr): υ_{max} 3040, 1693, 1659, 1595, 1570, 1533, 1400, 1369, 1285, 1267, 1107, 986, 851, 816, 756, 741.

HRMS (ESI): Calcd for $C_{29}H_{26}N_3O_4$ (MH^+) *m/z* = 480.1923, found *m/z* = 480.1930.

Glycylglycyl-β-naphthylamide hydrochloride salt 4.8



Fmoc-glycylglycyl-β-naphthylamide (330 mg, 80% pure, 0.55 mmol) was dissolved in 20% piperidine/THF (100 mL) and stirred at rt for 20 min. The solution was concentrated *in vacuo* to a volume of ~1 mL, diluted with THF (10 mL), and hexanes (100 mL) added with vigorous stirring. The resulting white precipitate was collected by filtration, washed with hexanes, then left at reduced pressure (0.03 mm Hg) for 2 d. This material was dissolved in 1:1 THF/1M aq HCl (10 mL) then concentrated *in vacuo* to leave the title compound in high purity as a white solid (121 mg, 75% yield).

Mp: 222-225 °C.

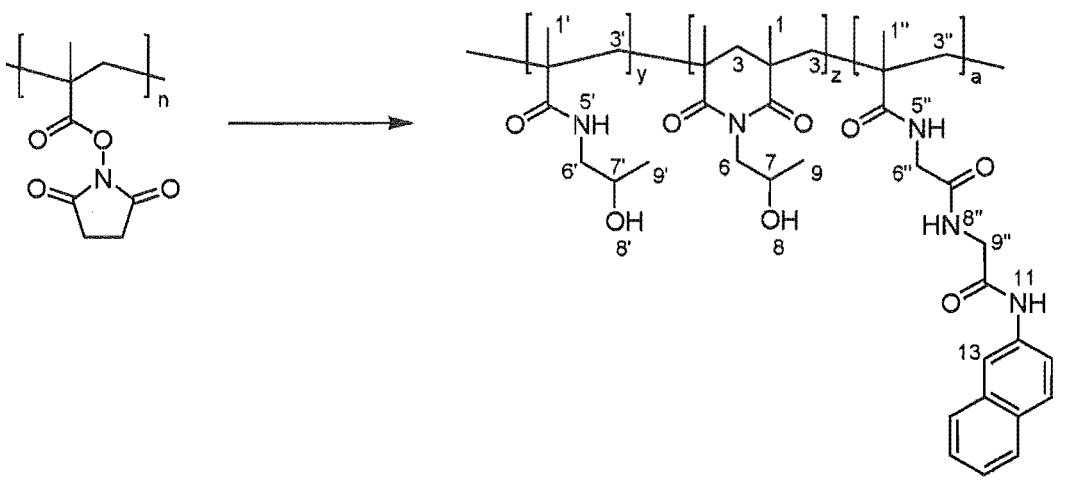
^{1}H NMR (500 MHz, d_6-DMSO): δ 3.66 (s, 1H, H-16), 4.07 (d, J = 5.9 Hz, 1H, H-13), 7.41 (td, J = 1.5, 7.6 Hz, 1H, H-8), 7.48 (td, J = 1.0, 7.3 Hz, 1H, H-7), 7.62 (dd, J = 2.0, 8.8 Hz, 1H, H-3), 7.80 (d, J = 8.3 Hz, 1H, H-6), 7.84 (d, J = 7.8 Hz, 1H, H-9), 7.87 (d, J = 8.8 Hz, 1H, H-4), 8.10 (s, 3H, H-17), 8.25 (s, 1H, H-1), 8.79 (, t, J = 5.6 Hz, 1H, H-14), 10.34 (s, 1H, H-11).

^{13}C NMR (75 MHz, d_6-DMSO): δ 39.9 (C-16), 43.0 (C-13), 115.5 (C-1), 120.2 (C-3), 125.0 (C-8), 126.7 (C-7), 127.5 (C-6), 127.7 (C-9), 128.7 (C-4), 130.0 (C-10), 133.6 (C-5), 136.6 (C-2), 166.7 (C-15), 167.7 (C-12).

IR (KBr): υ_{max} 2611, 1695, 1643, 1541, 1495, 1431, 1396, 1375, 1240, 1150, 980, 856, 820, 748.

HRMS (ESI): Calcd for $C_{14}H_{16}N_3O_2$ (MH^+) *m/z* = 258.1242, found *m/z* = 258.1238.

HPMA copolymer carrying glycylglycyl-β-naphthylamide[60]



A solution of pMAOS (100 mg, 546 μmol), naphthylamide **4.8** (14 mg, 55 μM) and TEA (15 μL, 108 μmol) in DMSO (400 μL) was stirred at under argon at 50 °C for 2.5 h. 1A2P (86 μL, 1.09 mmol) was added and the solution stirred for a further 1.25 h at 50 °C. The reaction mixture was subjected to LH20 chromatography, eluting with MeOH, and the high Mr material collected and concentrated *in vacuo* to give the copolymer as a clear, colourless glass (93 mg, 74% yield).

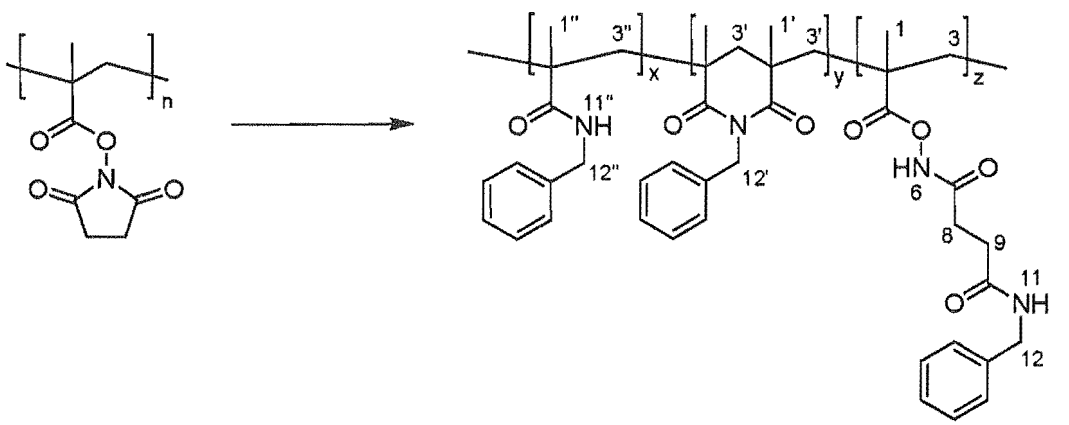
Following analysis by ^{1}H NMR spectroscopy, this polymeric material (90 mg, 391 μmol) was dissolved in DMSO (400 μL), 1A2P (86 μL, 1.09 mmol) added, and the solution stirred under

argon at 50 °C for 3 d. The reaction mixture was subjected to LH20 chromatography, eluting with MeOH, and the high Mr material collected and concentrated *in vacuo* to give the copolymer as a clear, colourless glass (55 mg, 97% yield).

^{1}H NMR (500 MHz, d_4-methanol): (all signals are broad) δ 0.9-1.5 (H-1, H-1', H-1", H-9 and H-9'), 1.5-2.4 (H-3 and H-3', H-3"), 2.9-3.3 (H-6'), 3.4-4.2 (H-6, H-6", H-7 H-7' and H-9"), 7.3-7.9 (H-5', H-5", H-8", H-11 and 6 × Ar), 8.1-8.3 (H-13). Integrals: H-1, H-1', H-1", H-9 and H-9' = 5.48; H-3, H-3' and H-3" = 2.00; H-6' = 1.05; H-6, H-6", H-7, H-7' and H-9" = 1.21; H-5', H-5", H-8", H-11 and 6 × Ar = 0.82; H-13 = 0.08.

IR (KBr): υ_{max} (all signals are weak) 2925, 1731, 1522, 1202, 1090, 948, 862.

HPMA copolymer carrying benzylamine



a) These protocols are an exact replication of those reported by Monge and Haddleton.[62] A solution of pMAOS (50 mg, 275 μmol) and benzylamine (60 μL, 549 μmol) in DMSO (300 μL) was stirred under argon at 50 °C for 5 h. The colourless solution was allowed to cool to rt then concentrated *in vacuo* (0.03 mm Hg for 7 d at rt). The white residues thus obtained were dissolved in DMSO (100 μL) and diluted with MeOH (10 mL). The solution was left at -20 °C for 3 d but no precipitation occurred.

b) The above protocols were repeated exactly, with the only difference being that, rather than attempted precipitation, the polymer product was purified by LH20 chromatography, eluting with MeOH.

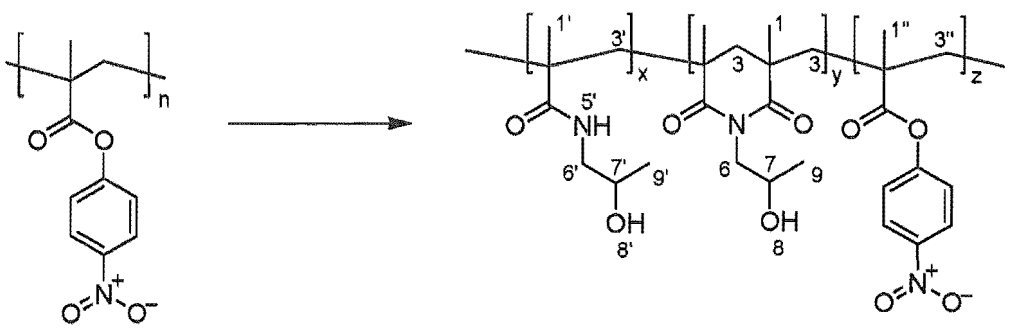
c) The above protocols were repeated exactly, with the only difference being that the polymer product was purified by LH20 chromatography, eluting with MeOH, immediately following the reaction, ie, without the 7 d under reduced pressure.

^{1}H NMR (500 MHz, d_6-DMSO): (all signals are broad) δ 0.6-1.4 (H-1, H-1' and H-1"), 1.4-2.2 (H-3, H-3' and H-3"), 2.3-2.5 (H-8" and H-9"), 4.0-4.4 (H-12"), 4.2-4.4 (H-12), 4.6-4.8 (H-12'), 7.1-7.3 (Ar), 7.7-8.1 (H-11"), 8.3-8.5 (H-11), 11.0-12.6 (H-6).

Construction of a calibration curve for *p*-nitrophenol on the HPLC

A small sample of *p*-nitrophenol was diluted 1:10 with MeOH, and aliquots (1, 2, 5 and 10 μL) of the resulting solution were injected onto the HPLC and analysed using the standard gradient elution protocols. All measurements were in close agreement and gave an average Peak $Area_{(315\ nm)} = 6.5 \times 10^{11}$ AU.min.mol^{-1}.

Reaction of poly(*p*-nitrophenyl methacrylate) (pNPMA) with 1A2P



A solution of pNPMA (20 mg, 97 μmol) and 1A2P (15 μL, 193 μmol) in DMSO (500 μL) was stirred under argon at 45 °C. Aliquots (5 μL) were periodically removed (at t = 0, 1.25, 3, 4.5, 7.5, 21 and 48 h), diluted into DMSO (1 mL), and an aliquot (10 μL) of the resulting solution injected onto the HPLC and analysed using the standard gradient elution prorocols. After 48 h at 45 °C, the solution was subjected to LH20 chromatography, eluting with MeOH, with the high Mr early-eluting fraction (which precipitated as it eluted from the column) being collected and concentrated *in vacuo* to give the copolymer as a white solid (10.3 mg, 84% yield).

^{1}H NMR (500 MHz, d_6-DMSO): (all signals are broad) δ 0.8-1.1 (H-9 and H-9'), 1.0-1.5 (H-1, H-1' and H-1''), 1.3-2.5 (H-3, H-3' and H-3''), 2.8-3.1 (H-6'), 3.4-3.9 (H-6, H-7 and H-7'), 4.4-4.8 (H-8 or H-8'), 4.5-4.8 (H-8 or H-8'), 7.3-7.6 (H-5' and PNPMA Ar), 7.6-7.8 (end group Ar), 8.2-8.4 (end group Ar), 8.3-8.4 (PNPMA Ar).

Poly(methacryloyl chloride) (pMAC)

This was synthesised from methacrylorl chloride following the slightly modified procedure of Yamaguchi and Gibson.[132] AIBN (9.0 mg, 56 μmol) was weighed into a round-bottomed flask, methacryloyl chloride (1.00 mL, 10.2 mmol) and toluene (1.80 mL) added, a reflux condenser fitted, and the solution degassed with 3 successive freeze-pump-thaw cycles. The solution was then stirred under argon at 85 °C for 21 h. The solution was allowed to cool to rt, then hexanes (20 mL) added with vigorous stirring and the resulting white precipitate collected by filtration, washed with hexanes and dried *in vacuo* to give the polymeric product as a white powder (296 mg, 28% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ (signals are broad and 'spikey') 1.2-1.5 (CH_3), 2.2-2.4 (CH_2).

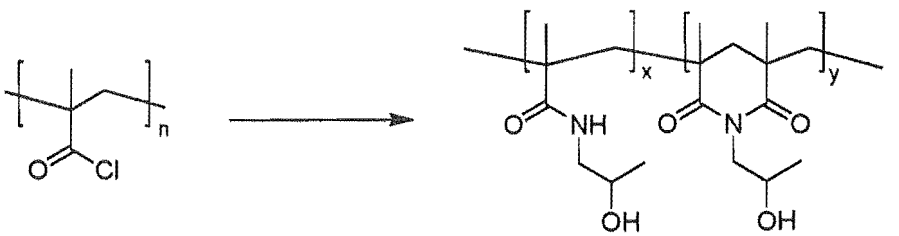
Poly(methyl methacrylate) (pMMA)[132]

A solution of pMAC (40 mg, 0.38 mmol), MeOH (700 μL, 17.3 mmol), and pyridine (145 μL, 1.8 mmol) in THF (7.0 mL) was refluxed under argon for 3 d. The colourless solution was allowed to cool to rt then concentrated *in vacuo*. The residues thus obtained were dissolved in

1:1 THF/MeOH (3 mL), MeOH (20 mL) added, and the resulting white precipitate collected by filtration and dried *in vacuo* to give the polymeric product as a white powder (9 mg, 24% yield).

^{1}H NMR (500 MHz, $CDCl_3$): δ (signals are broad and backbone signals are 'spikey') 0.8-2.1 (5H, backbone CH_3 and CH_2), 3.5-3.7 (2H, OCH_3).

Reaction of pMAC with 1A2P



pMAC (20 mg, 0.19 mmol) was weighed into a Young's tube and placed under argon. 1A2P (76 μL, 0.96 mmol), TEA (40 μL, 0.29 mmol) and DMSO (0.5 mL) were added and the resulting solution left in the oven at 70 °C for 16 h. The solution was allowed to cool to rt, diluted with MeOH (0.5 mL) and subjected to LH20 chromatography, eluting with MeOH, with the high Mr fraction being collected and concentrated *in vacuo* to give the polymeric product as a clear colourless glass (23 mg, 96 % yield).

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APPENDIX I: HPLC RETENTION TIMES

Compound	Retention time (min)	Elution protocol*
Variolin B	8.1	Standard gradient
DVB	7.3	Standard gradient
2.16	10.2	Standard gradient
2.16	3.0	85% MeOH/H_2O
FmocGFLG	15.3	Standard gradient
2.18	19.3	Standard gradient
2.18	8.6	85% MeOH/H_2O
2.19	5.0	85% MeOH/H_2O
2.20	4.3	85% MeOH/H_2O
2.27	11.4	Standard gradient
FmocGly	13.6	Standard gradient
2.28	15.1	Standard gradient
2.29	9.9	Standard gradient
2.32	10.7	Standard gradient
2.33	11.6	Standard gradient
2.34	15.8	Standard gradient
3.26	10.2	Standard gradient
3.26	9.6	60% MeOH/H_2O
4.8	8.8	Standard gradient
p-nitrophenol	11.6	Standard gradient

* See General experimental for the standard gradient elution protocol